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THE GEORGE WALD

Proctor Medal Award

PROCEEDINGS

of the

Association for Research in Ophthalmology, Inc.

Twenty-fourth Meeting

Atlantic City, New Jersey

June 7, 8, and 9, 1955

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For a complete table of contents see page one

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REMARKS ON ACCEPTANCE OF THE PROCTOR MEDAL AWARD

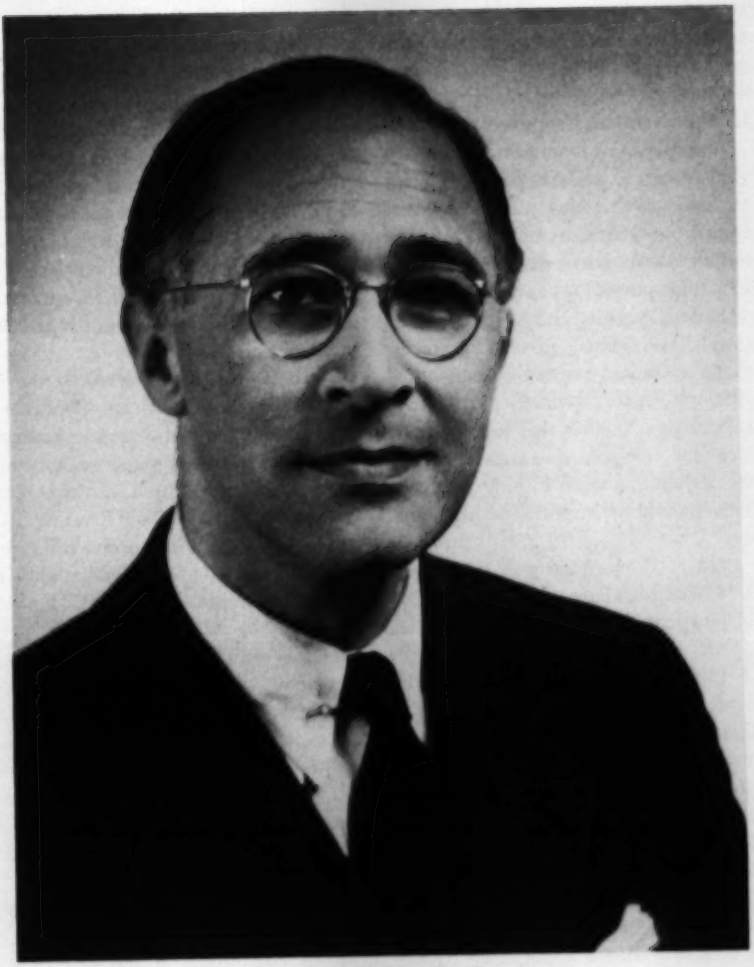
June, 1955

GEORGE WALD, PH.D.

Having had the opportunity elsewhere in these *Proceedings* to discuss the work of our laboratory, nothing remains but to express my deep appreciation for the Proctor Award. I have spent 28 years with the eye and vision, having been introduced to both as a graduate student by Selig Hecht. Hecht was a great teacher and physiologist. Also he was one of those rare persons who sets a standard, both at work and at leisure. I was fortunate in having his instruction, and later his friendship. I saw all too little of him after leaving his laboratory but I felt his presence always. What I did or said or wrote was in a sense always addressed to him; and having become used to this while he lived, it didn't change very much afterward. I should like to think that in awarding the Proctor Medal to me, you honor his memory also; and wish with all my heart that his untimely death had not deprived you of the opportunity to honor him in person.

I should like to accept the Proctor Award also in part on behalf of my young associates. Since the end of the war a small group of us has worked closely together: Ruth Hubbard, Paul K. Brown, and more recently Patricia H. Smith. Other workers have made notable contributions, but these associates have formed the nucleus of the laboratory, and most of our work has come from them.

(At this point Professor Wald, by request, spoke on the origin of life. He has not submitted his remarks for publication here, but they followed substantially his article on this subject which was published in the August, 1954, issue of the *Scientific American*.)



GEORGE WALD, PH.D.

BIOGRAPHICAL NOTES

GEORGE WALD, PH.D.

George Wald was born in New York City on November 18, 1906, and was educated in the New York public schools. He received his Bachelor of Science degree from New York University in 1927, majoring in zoology. He then undertook graduate work at Columbia University and received the Ph.D. in 1932. During his graduate period he was a student and research assistant of Prof. Selig Hecht, an association which was most fruitful personally and professionally. His research during these years dealt with problems of visual physiology and his thesis analyzed the visual acuity and brightness discrimination of the fruitfly, *Drosophila melanogaster*. As a student he had a keen interest in the physical sciences, particularly physical chemistry, but he also felt the need to explore the integrated functioning of organisms, a dual approach to biologic problems which has been implicit in his work ever since.

From 1932 to 1934, Wald was a National Research Council Fellow in Biology. The first year, he spent abroad in the laboratories of Prof. Otto Warburg, Prof. Paul Karrer, and Prof. Otto Meyerhof; the second at the Department of Physiology of the University of Chicago. While in Professor Warburg's laboratory in Berlin-Dahlem, Wald first found vitamin A in the retina. Vitamin A had just been isolated in the laboratory of Professor Karrer in Zürich and Wald went there to complete the identification of retinal vitamin A. This marks the beginning of his analysis of the biochemistry and physiology of vision in terms of the metabolism of the visual pigments and the vitamins A—the work for which he is best known.

Dr. Wald came to Harvard in 1934 as tutor in Biochemical Sciences and has been there ever since: as Instructor and Tutor in Biology (1935-39), Faculty Instructor (1939-44), Associate Professor (1944-48), and Professor of Biology (1948-). He was elected to the National Academy of Sciences in 1950, and is a Fellow also of the American Academy of Arts and Sciences. He is Chairman (1954-55) of the Divisional Committee on Biology and Medical Sciences of the National Science Foundation, and a trustee of the Marine Biological Laboratory in Woods Hole. He is a member of the American Society of Biological Chemists, the American Physiological Society, the Optical Society of America, the Association for Research in Ophthalmology, Sigma Xi, the Society of General Physiologists, and the American Association for the Advancement of Science.

In 1939, Dr. Wald received the Eli Lilly Award of the American Chemical Society for "fundamental research in biochemistry," and in 1953 he was given the Lasker Award of the American Public Health Association "in recognition of his outstanding discoveries in biochemistry with special reference to the changes associated with vision and the function of vitamin A." In 1952, he was a national Sigma Xi lecturer.

Dr. Wald's research has been concerned primarily with the biochemistry of vision. After finding vitamin A in the retina, he discovered a new carotenoid, retinene (later identified by Morton as vitamin A aldehyde), and proceeded with a systematic elucidation of the role of retinene and vitamin A in the synthesis and bleaching of the visual pigments.

Boll (1876) had discovered the first visual pigment, rhodopsin, and Kühne (1878) had hinted at the existence of porphyropsin. Wald and his group have extensively studied both of these rod pigments and have also identified and characterized two cone pigments—iodopsin and cyanopsin. They have succeeded in synthesizing all four from their components—carotenoid and protein—and have shown an underlying similarity in their structure and behavior. Re-

cently, with Paul Brown, Wald set up a model for the manner in which the bleaching of a visual pigment might elicit a nervous impulse.

The isolation of vitamin A from retinas brought with it the identification of a new vitamin A, vitamin A_2 and of retinene$_2$. This opened up another realm of Professor Wald's research activities: biochemical evolution. He has explored the distribution of the vitamins A_1 and A_2 in the animal kingdom and shown a transition from A_2 to A_1 as vertebrates left fresh water either for the dry land or the ocean. With Dr. Riggs and others, he has also explored the evolution of the hemoglobins and other respiratory pigments. He has discussed these and other molecular changes in several lucid essays on biochemical evolution. More recently he has also been interested in the origin of complex organic molecules and of organisms.

His research in vision, apart from its chemical aspects, has dealt with dark adaptation and night blindness, spectral sensitivity functions, the chromatic aberration of the human lens, and the contributions of physiologic filtering mechanisms by lens and macular pigmentation to vision. With Dr. Auerbach he has recently started an analysis of human color receptors. He has also begun to develop a theory of the visual threshold which will account for the large changes in visual threshold which accompany relatively small changes in the concentration of visual pigment.

Apart from the breadth and high quality of his research, Wald's chief scientific contribution has been as lecturer and teacher both in his special field and on broader topics of current biological thinking. He has never been satisfied with the mere discovery of new facts, but has always insisted on exploring the framework which gives them more general meaning.

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A NEW INTERPRETATION OF THE FUNDUS REFLEXES

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For many years we have taught that the bright reflections coming from the fundus are, in the majority of cases, reflexes from the posterior surface of the vitreous and not from the retina. Reflex as here used is not in the physiologic sense of an involuntary motion, but as the technical, descriptive of reflected light.

Any trained ophthalmoscopist can by means of stereo-ophthalmoscopic investigation or stereophotographs confirm the statements that are made here. Some may say that the photographs are not true scientific stereoscopic reproductions, we remind them that much of stereopsis is from brain stimulation and less from actual depth portrayal.

The nonpathologic fundus reflexes, more common and large in the young, depend upon the source and intensity of the ophthalmoscopic light, the angle of incidence by which viewed, the size of the pupil, the refractive error and color of the background, and the age of the patient. The reflex will move or fade from view when the position of either the patient's eye or the examiner is changed. Incidentally black and white photographs are often better than the colored for this type of study.

A few photographs are presented to focus attention on the source of the reflexes and stress the value of accurate localization as a means of more exact diagnoses between physical phenomena and pathologic alterations.

For more than 60 years authors have continued the expression "retinal reflex," although some of the keen observers on occasion have referred to that reflex as not coming from the retina or blood vessels.

Haab wrote: "It is true that in dark eyegrounds, especially in young individuals, a small, bright sickle or ring is seen, due to the reflection of the light at the bottom of the fovea centralis, and although this reflex is a little in front of the retina the difference is so slight that it need not be considered in calculating the refraction."

From the same author we find in another place: "Occasionally we see an additional somewhat larger luminous ring concentric with the fovea reflex."

Dimmer decided that "the macular reflex is produced by the concave cylindrical or sphero concave surfaces found on the inner surface of the retina." He also stated, that "the bright stripe along the center of the vessel is due in case of the arteries to the reflection of the light by the blood corpuscles in the axial stream and in the case of the veins to a similar reflection by the anterior surface of the blood column." Those who are interested will refer to the article by Wilmer and the discussion which followed his presentation.

The preretinal reflexes may be conveniently segregated into those anterior to the fovea, in front of the macula, following the course of the temporal vessels, less often the nasal ones, and least frequently over the disc itself. Occasionally they appear as minute, pinpoint, sparkling specks.

By studying normal fundi it is comparatively easy to prove that the bright or smoky dot, small arc, or smudge which overlies the fovea is anterior to the retina. Convincing proof that it is not foveal is found in a circumscribed hemorrhage over the macular region where the distinctive spot is present although the fovea is hidden beneath the blood.

The perimacular reflex may be an isolated, sharp line overlying the margin of the dark macular region or a band or a broad encircling ribbon and may have an uneven surface. It may look like a cloud extending beyond the temporal or nasal margin of the macular zone, a single arc, or one above and another below with an infinite variety of

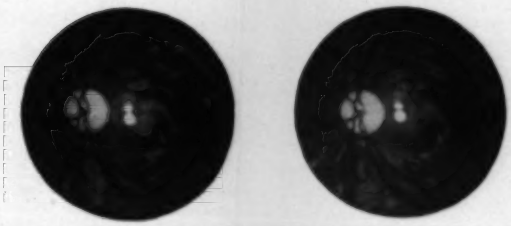

Fig. 1 (Bedell). The fundus of an 18-year-old boy with traumatic optic atrophy. An excellent example of widely dispersed, sharply delineated reflexes from the vitreous. (Stereophotographs.)

sizes and densities widely disseminated. A common distribution is in front of the temporal vessels. This may be limited to either the upper or the lower branches or cover both. Similar reflections on the nasal side are neither from the vessels nor the retina and are only seen over the designated regions. Occasionally there are a great number of gray flecks not limited to the perimacular or prefoveal situations. All of these reflexes originate on the posterior surface of the vitreous, sometimes a considerable distance in front of the retina although they may be close to the retinal plane. The exact distance cannot be measured except stereoscopically and for that reason photographs supply sufficient data from which to draw conclusions.

The true retinal reflections come from the differences in physiologic level, increased visibility or anomalous changes such as congenital defects, or from pathologic alterations in the fundus.

About the macular region and sometimes so thin that they may be mistaken for reflexes are the very delicate, thin veils, skeins, or nets which are found in hypertensives, some nephritics, occasionally in diabetics, and not rarely after thrombosis of the central retinal vein. They may be silvery, gray, or even yellow and careful inspection proves that they are not displaced when viewed at different angles and that therefore the characteristic dispersion of the preretinal reflex is absent. It is rarely difficult to distinguish between the translucent reflexes and epipapillary membranes; those vestiges of embryologic life which are always attached to the nasal side of the center of the disc. Very small drusen may look like veils.

The differential diagnosis between partial stellate figures in the macula and reflexes from that region seldom, if ever leads to uncertainty or calls for discussion, for the dots which make up the spokes are usually distinctly discrete.

Retinal edema may be mistaken for a reflex, particularly when close to the disc. Edema as in traumatic retino-angiopathy may delude the careless observer into con-

Fig. 2 (Bedell). The left eye of a 32-year-old woman, with a −3.0D. sph. ⌒ −0.5D. cyl. ax. 180° = 20/20+. The soft gray reflex between the macula and the disc comes from the posterior vitreous layer. The one over the fovea is anterior to the fovea and from the same layer. (Stereophotographs.)

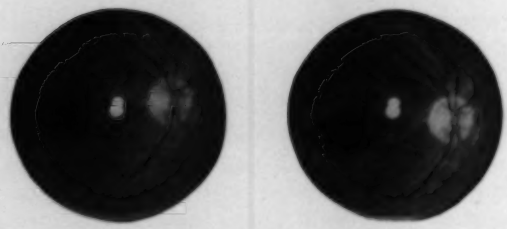

Fig. 3 (Bedell). Stereophotographs of a 20-year-old woman. Central vision of 4/200, optic atrophy, and diffuse circummacular reflexes from the posterior layer of the vitreous simulating in appearance retinal exudates.

sidering the clouds as reflexes similar to those that come from exudates.

The reflexes from the blood vessels offer some interesting suggestions for debate. Those on the surface of a retinal vein may be small, isolated, lustrous dots, spots, oblongs, or long, narrow stripes, which invariably shift when the vessel pulsates. It is well known that retinal artery pulsation is rare, so that if the veins and arteries are of approximately the same size and color as in some cases of polycythemia the vacillating lights usually come from the veins. In considering the reflexes near or on the veins and arteries special attention is drawn to the paralleling bright lines which do not arise on the vessel walls but are separated from them. These are most easily seen in cases of terminal central retinal artery embolism as streaks.

Care must be taken to recognize direct illumination in contrast to the preretinal and retinal reflexes. The mere mention of the universally appreciated changes in the artery alluded to as copper wire, silver wire, and atherosclerosis is sufficient to attract attention to the lighting of the fundus opposed to reflections from it.

The luminous, arcuately distributed retinal fiber may at times suggest overlying prevascular reflexes for both follow the same general course.

Reflections may come from plaques of cholesterin crystals with their iridescent sheen.

The reflexes from retinitis proliferans strands are by their very location easily appreciated as is the glistening, convex top of central serous retinopathy.

From these provocative suggestions it should be evident that the fundus reflexes have been too long neglected in the discriminating differentiation of one from another and that they have not been subjected to critical analyses. The recognition of the preretinal reflexes from the retinal and both from the direct fundus illumination is a stimulating ophthalmoscopic exercise recom-

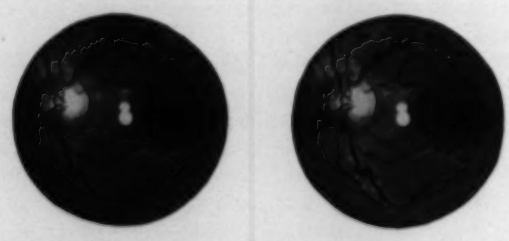

Fig. 4 (Bedell). Stereophotographs of a 20-year-old woman. The reflexes are about but not over the tortuous vessels.

mended to all who wish to excel in the interpretation of the intraocular signs of the disease.

344 State Street.

Discussion

Dr. P. Robb McDonald (Philadelphia, Pennsylvania): It has been said that keen observation maketh a good clinician. I am afraid I am a horrible clinician. I misunderstood the time of the meeting as two o'clock instead of one; hence, I was not here to discuss Dr. Bedell's paper. I want to apologize to him and to the members of the association.

I had occasion to read the paper, and I think Dr. Bedell has pointed out to us a most interesting phenomenon, which most of us see every day but pay little attention to. He believes (and, I think, quite rightly) that most of the reflexes we have called retinal reflexes or physiologic reflexes are due to the posterior cortical layer of the vitreous body.

Those of you who have followed Schepen's work know that, in the frozen sections of eye, he has been able to demonstrate very clearly that there is a posterior cortical layer to the vitreous.

This physiologic reflex is not a true retinal reflex, but is a preretinal reflex. It is distinguished from the retinal reflexes by the fact that it is evanescent. It may disappear or change as one changes illumination in looking at the fundus. One may also get a retinal reflex when there is some difference in the surface of the retina, with physiologic elevation or depression.

Dr. Bedell rightly points out that many of the reflexes which we describe are not retinal reflexes or, if they are, they are from pathologic changes in the eye. He showed some beautiful pictures demonstrating these. One can have, of course, the reflex of the retinal vessels which occurs when one has cirrhosis. One can get some edema showing strands of reflex around the raised area; and one can get that so-called succulent appearance around the posterior pools following some of the vascular accidents.

I think Dr. Bedell has done us a service in pointing out the etiology of the reflexes we see. Some of them are from the posterior choroidal area of the vitreous; and some of them are true reflexes. They are not one and the same thing.

GONIOCYCLOSCOPY AND OPHTHALMOSCOPY*

With slitlamp and Allen-Thorpe gonioprism

Harvey E. Thorpe, M.D.
Pittsburgh, Pennsylvania

This paper deals with a simple method for visualizing the lens equator, ciliary body, zonule of Zinn, central and peripheral retina, while utilizing the advantages of true slitlamp microscopy.

Brief historical review

The first description of a clinical method for examination of the anterior chamber angle, the retinal periphery, and the inner surface of the ciliary body was reported by A. Trantas[1] in 1900, and again in 1907, and 1918. He used the terms gonioscopy and cycloscopy in his later articles. His method is now well known. (It consists of making this region accessible to view by pressure on, or indentation of the ciliary region of the eyeball, with a blunt instrument, or by pressure of the examiner's finger transmitted through the eyelid.)

Koeppe[2] in 1922 described the use of a flat corneal contact lens for slitlamp microscopy of the deep vitreous and retina. Lindner[3] reported on a monocular offset microscope which he used with Koeppe's flat contact lens for slitlamp examination of the deep vitreous. von Sallmann and Rieger[4] in 1934 reported their observation of vitreous detachment in several cases of retinal separation with the aid of the Koeppe lens and Lindner monocular microscope. In 1936, Thorpe[5] modified Koeppe's flat corneal contact lens for deep vitreous and retinal biomicroscopy by enlarging its outer surface from 8.0 to 14.7 mm. in diameter and making that surface convex (+5.0D.). This

* From the Montefiore Hospital.

avoided annoying reflections, and, because of its larger front surface, more of the fundus could be seen with the biomicroscope. Kronfeld found this to be helpful for peripheral fundus studies in retinal detachment. Numerous modifications of the flat lens followed. In 1938, Goldmann[8] further improved this lens by putting a black hood on it. This protected the front surface of the lens against being smeared by lid blinking. This lens with the Haag-Street slitlamp employing a reduction prism, removed the difficulties which had beset slitlamp examination of the fundus.

Hruby[7] further simplified the deep vitreous and fundus biomicroscopy in 1941, by employing a −55.0D. preset lens with focal illumination of the slitlamp. In 1950, he published a comprehensive book on this subject. Lemoine and Valois had tried a similar strong minus preset lens for fundus study a quarter century earlier, but they employed diffuse illumination which gave little additional information. In 1949, Goldmann[8] described a three-mirror contact lens for fundus examination. Braley and Allen[9] in 1951 reported a single flat surface contact prism and a separate flat surface contact lens for the respective slitlamp examination of the peripheral and central retinal areas.

In 1953, Allen, Braley, and Thorpe[10] reported at the A.M.A. section at its New York meeting the construction and use of a four-mirror gonioprism which was mounted in a simple acrylic speculum. This gonioprism has simplified the technique of slitlamp examination of the lens periphery, the suspensory ligament, and neighboring structures as reported[11] to the East Central Section of this association in a paper read at its Pittsburgh meeting in January, 1954. This application of the gonioprism was particularly significant because the equatorial region of the lens could in most instances be studied through the normal pupil aperture, while one was performing a routine gonioscopy. There were some limitations to this method in observing the horizontal meridian of the nasal and temporal angles, which were overcome by simply rotating the handle of the instrument through an arc of 40 to 50 degrees in the temporal direction. As I[10] have previously reported, it was possible to broaden the scope of the internal eye examination by dilating the pupil to permit slitlamp ophthalmoscopy of the central and extreme peripheral areas and, under special conditions, also the ciliary body.

N. Trantas[12] in 1947 reported on biomicroscopy of the lens zonule in normal eyes and, in 1949, on slitlamp studies on ciliary body cysts in unoperated eyes. He used the Koeppe gonioscopic contact lens with maximal pupil dilatation produced by atropine and subconjunctival injection of adrenalin for study of the posterior chamber through the iridophakic cleft. Scheie[13] in 1954 reported in an excellent article on gonioscopic observation of iris cysts and tumors in the posterior chamber by a similar technique. Reese[14] and Dunnington[15] (quoted by Reese) have noted cysts of the ciliary body and processes on clinical and histologic examination. Goldmann[8] and François[16] have studied the ciliary body and the peripheral retina with the Koeppe or Goldmann contact lens. Experience with the four-sided Allen-Thorpe gonioprism has shown it to be much simpler in operation in the routine performance of clinical slitlamp examination of lens periphery, ciliary body, and retina than either of the above methods.

Technique

The routine technique for gonioscopy is employed. The eyes are anesthetized by the instillation of two drops of Tetracaine (0.5 percent). The patient is seated at the slitlamp. The gonioprism with a drop of one-percent methyl cellulose in the corneal face is inserted between the eyelids. (I employ either Goldmann-Haag Streit model or the Zeiss-Opton slitlamp because their joy-stick principle and universal elevating mechanism permits easy operation of the instrument with one hand while the other hand supports the gonioprism.) The smallest angle possible illuminating beam and microscope axis also

makes the newer instruments—including the Aimark slitlamp—preferable to the prewar instruments.

Topography

The normal structures to be identified from anterior to posterior with maximally dilated pupil reflected in the respective gonioprism mirror are:

1. Concave corneal dome
2. Corneoscleral transition zone and corneal wedge (Busacca)
3. Annular line of Schwalbe
4. Trabeculum and region of Schlemm's canal
5. Scleral spur
6. Anterior chamber portion of ciliary body
7. Peripheral iris processes
8. Iris
9. Pupil border
10. Posterior chamber through iridophakic cleft
    a. Abnormal protuberance, cysts, tumors, foreign bodies on posterior iris surface which encroach on the posterior chamber
    b. Ciliary processes
    c. Anterior interciliary fibers of zonula
11. Anterior lens capsule
12. Lens equator
13. Triangular perilental space (Hanover's canal) with occasional zonular fibers
14. Posterior leaf of zonular membrane with incorporated lens fibers
15. Wieger's hyaloideocapsular ligament
16. Pars plana or orbiculus ciliaris
17. Perilenticular anterior hyaloid
    Insertion of posterior zonular fibers into hyaloid
18. Vitreous base (seen only with complete iridectomy in intracapsular lens extractions)
19. Ora serrata (seen with difficulty in noniridectomized eyes)
20. Peripheral retina
21. Posterior hyaloid if detached

Through the central corneal contact portion of the gonioprism one can readily observe the posterior hyaloid with the wider angle between beam and microscope and the macular and perimacular retina in optical section or by other methods of slitlamp illumination.

Posterior chamber

The posterior chamber can only be seen with the maximally dilated pupil (eight mm. or greater). In the normal eye with an axial chamber depth of two to three mm., a visible cleft then appears between the posterior pupil border and the lens capsule. The iridophakic cleft permits a view of the posterior chamber contents as they are reflected in each of the mirrors of the gonioprism. The examination is made with the patient sitting at the slitlamp. Diffuse and focal illumination are employed. Retroillumination is also tried, but rarely gives definitive information.

In the unoperated eye one may note protuberances of the iris into this space such as tumors, cysts, or foreign bodies. A vitreous hernia may be recognized. Changes may be observed in the ciliary processes with the scanning beam one may see individual zonular fibers as they traverse the valleys between the ciliary processes.

Case 1

Mrs. J. A., aged 58 years, had been seen in March, 1954, with early cupuliform lens opacities in the right eye. In January, 1955, she returned with partial posterior displacement of a now advanced cataract in that eye with some iris bulge in the inferotemporal quadrant. Examination with the gonioprism of the retro-iridic area revealed a lobular nontransilluminating darkly pigmented mass. Histologic examination proved this to be a cyst. Biomicroscopic examination gave valuable descriptive information, but failed to make a definitive diagnosis.

Case 2

R. S., aged 41 years, a man, was seen on October 22, 1954. He suffered an injury of

the right eye when striking a chisel with a hammer. There was a small limbal perforation at the 7-o'clock position. X-ray examination by Vogt's soft-tissue technique revealed a small foreign body. Examination of the posterior chamber with the gonioprism revealed a small splinter wedged between two ciliary processes at about the 7-o'clock position. The Berman locator test was positive. The steel splinter was brought into the anterior chamber with the giant magnet and then removed through a corneal incision with a small hand magnet. The splinter measured 1.0 by 0.5 by 0.3 mm. Final vision was 20/30, O.D.

Lens periphery, zonula, and Hanover's canal

The lens equator and suspensory ligament are ordinarily accessible to observation only through an iris coloboma, in aniridia, and in subluxatio lentis. With the gonioprism one can observe them with the naked eye, the loupe, or the biomicroscope. The sharp narrow focal beam, retroillumination, specular reflection, and transitional illumination are employed for the examination of the lens periphery. Observation and illumination are by way of the gonioprism mirror surfaces.

While performing routine gonioscopy in eyes with undilated pupils and the gonioprism in normal position the upper and lower lens equatorial region, the site of Wieger's hyaloideocapsular ligament, the circumlental space, identified as Hanover's canal, and the posterior leaf of the zonule can be observed biomicroscopically in a patient whose anterior chamber is between one and two mm. in axial depth. The pupil diameter need not be larger than three to four mm. It is thus possible to examine eyes in which mydriasis is contraindicated.

Pupil dilatation becomes necessary as a rule for equatorial lens study in cases with an axial anterior chamber depth exceeding three mm.

The lens phantom appears sharply flattened posteriorly when viewed in focal light in the reflecting surface of the gonioprism. This is due to the high index of refraction of the lens nucleus periphery which blurs the focus of the focal beam and sharply refracts the light beam which passes quite obliquely through it.

Normally, Wieger's ligament can be seen by focal illumination or in specular reflection about 1.5 to 2.0 mm. within the equator circle at the posterior capsule. It appears as an undulating somewhat flattened circular band about 0.3 mm. wide under ×20 magnification. Groups of zonular fibers in two or three can be seen to lie in the posterior zonular membrane as it approaches its insertion into the posterior capsule.

A case of eyeball concussion had a small hyphema. Posteriorly the blood was delimited from passing up into Berger's space behind the lens at the site of Wieger's ligament.

I have observed formed elements (evidently white blood cells) in the anterior vitreous just posterior to this zonular leaf in cases of uveitis and mild cyclitis. Only on rare occasions are they visible in Hanover's canal. This may account for the infrequency of complicated cataract in most cases of uveitis. Hanover's canal can be visualized in the upper and lower quadrants only as a rule with the vertical beam. With the horizontal beam in the Zeiss-Opton instrument it has been seen with difficulty with the gonioprism at an angle of about 30 degrees. Early peripheral lens opacities in form of dots and spokes can be seen near the equator.

A zonular separation was seen as such. One could see a vitreous hernia into the posterior chamber and through the iridophakic cleft. To repeat, the above structures are visualized only by reflection in the gonioscopic mirror.

Peripheral ophthalmoscopy and posterior cycloscopy (orbiculus ciliaris)

Focal, proximal, and retroillumination are the methods employed in the slitlamp exami-

nation. Peripheral ophthalmoscopy with gonioprism and biomicroscope can be performed in a limited way only with the undilated pupil. Maximum mydriasis is advisable. Visualization for peripheral areas is necessarily by reflection in one of the gonioscopic mirror faces.

The extent of the retinal periphery which can be visualized in a patient without iridectomy and lens in situ possessing clear media, varies to some extent inversely with the axial chamber depth. The peripheral lens region, particularly the edge of the highly refractile lens nucleus, may blur the image. However, in the large majority of cases, it is possible to do adequate slit ophthalmoscopy and deep vitreous examination. Retinal tears may be visualized in the reflecting goniosurface. The vitreous bands responsible for dragging on the retina may also be seen. This is especially true at the present time for nearly two thirds of the upper hemisphere and also for the same fraction of the lower periphery. This is accomplished by doing part of the examination with the prism handle vertical, utilizing the upper and lower mirrors. The instrument handle then is swung laterally 45 degrees and one carries on the examination in two adjacent pairs of mirrors in sequence until the circumference is examined. The mirror surface reflects the zone between the equator and ciliary processes. This can be enhanced by elevating and depressing the gonioprism handle. The lens periphery and deep chamber usually block out the region of the ora serrata. It is likely that the addition of an offset horizontal beam to our equipment might improve the scope of visualization.

The aphakic eye with dilatable pupil and particularly with complete iridectomy permits an excellent view from the chamber angle to the equator in the region of the iridectomy. One can study most of the details of the zonular membrane and satisfy himself that he is actually dealing with such a structure. This membrane is readily visible in 90 percent of iridectomized eyes in the sector of iris excision. One can follow the slitlamp beam and optical section in the uncomplicated case over the superficial grayish cell layer of the ciliary processes and identify the structures in the following order from anterior to posterior beginning with the scleral spur as seen in the lower gonioprism mirror between the pillars of the iris coloboma:

1. Corpus ciliaris
2. Remains of iris root
3. Ciliary processes—club-shaped and wafer-shaped, and in some cases like a short cock's comb arranged in corona form. (Sometimes, one observes small thickenings and, more rarely, cysts on the ciliary processes.)
4. Occasional remains of individual fibers arising in the valleys between processes
5. Zonular membrane is one to two mm. wide, having two or three broken concentric lines. The membrane ends in a ring composed of:
6. Remains of a thickened, firm Wieger's ligament.
7. Vitreous face (anterior hyaloid) herniating forward and indented by Wieger's ligament (seen with focal beam). (One can then follow the vitreous posteriorly along the pars plana of the ciliary body.)
8. Occasional zonular fibers inserted into hyaloid two or more mm. posterior to its indentation.[7] They may be seen by oscillating and transitional illumination.
9. The orbiculus ciliaris is light-brown in appearance and adjoins the dark-brown region near the dentate ora serrata.

At this point, for a width of about 1.5 to 2.0 mm. one can often visualize

10. The base of the vitreous (just anterior to the ora junction with the retina). In many cases the vitreous is collapsed.

11. The posterior hyaloid may hang down from the vitreous base or be reflected back onto the retina and hang from there.
12. The extreme anterior portion of the retina appears grossly without blood vessels. Near the ora it appears as a faint gray spawnlike surface for a width of a mm. (×20 magnification). This corresponds to the region of Blessig cysts seen histologically in the retinal periphery.
13. The peripheral retina with thin apparently terminating blood vessels.

Areas of chorioretinal change (pigmented or atrophic) may be seen in some eyes. In diabetic retinopathy a somewhat elevated pale moundlike ring running just back of and concentric with the ora was observed in a number of cases.

The pathologic aberrations of minor and major complications of ocular surgery can be observed. In most of the above it is possible to obtain a shallow or somewhat deeper optical section of the translucent tissues. Early or late detachment of the retina, the vitreous, retinal holes, and other pathologic conditions are readily studied.

Central slitlamp ophthalmoscopy

This is accomplished through the center of the gonioprism and done preferably through a dilated pupil. Focal, proximal, and retro-illumination are used. This method supplants the Hruby lens and gives a more brilliant picture. It corresponds to the flat contact lens method. An area about five or more disc diameters across can be observed. This to some extent depends on the diameter of the corneal contact surface and on the refraction.

For study of the deep vitreous and vitreous detachment a 15 to 20-degree angle is chosen between the axis on the slitlamp beam and that of the microscope. This prevents the light reflected from the portion of slit-illuminated retina from interfering with vitreous examination. A positive flare can often be seen in the posterior vitreous in the path of the slitlamp beam in uveitis and cyclitis.

Cellular exudation can be observed; also, a conglomeration of groups of cells in the form of precipitates can be readily observed on the posterior hyaloid in granulomatous uveitis.

Hemorrhages into the vitreous or between vitreous and retina or within the retina itself can readily be seen.

Optic papilla and retina proper

Focal and proximal illumination is employed with the narrow beam. A narrow angle (five to 10 degrees) between illumination and microscope axis is necessary so that binocular vision can be used for observation.

The superficial translucent tissues of the papilla can be examined in optical section and also by proximal illumination.

The retina proper can be studied in optical section, by proximal illumination and by transmitted light. This applies to the macular and extramacular region. Cysts, holes, hemorrhages, microaneurysms, edema, infiltrations, atrophy, detachment, and neoplasms can be studied and differentiated.

Case 3

Mrs. H. R., aged 26 years, was recently seen in consultation. She gave a history of frequent recurrence of macular edema in the right eye. Routine ophthalmoscopy seemed to confirm the diagnosis. However, slitlamp ophthalmoscopy through the gonioprism showed circumscribed macular edema with flat detachment of the retina and irregular exudate on the outer retinal surface. Further examination with the gonioprism mirror surface revealed a retinal disinsertion in the temporal periphery.

Summary and Conclusions

The four-mirror Allen-Thorpe gonioprism permits ready examination of the anterior-

chamber angles, the lens periphery, the posterior chamber, the zonule of Zinn, and the ciliary body under special conditions by reflection in the mirror surfaces.

The retinal periphery can be studied in focal light and by other means of illumination while viewing structures in the reflecting mirror through a dilated pupil.

The central portion of the gonioscope serves as a corneal contact lens for examining the macular and perimacular areas of the retina and of the optic papilla. It can be used with the focal beam of the slitlamp to study the deep vitreous as well as the retina.

Slitlamp microscopes using the joy-stick principle are of considerable help in performing the examination.

Jenkins Building (22).

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Discussion

Dr. Hermann M. Burian (Iowa City, Iowa): I am not prepared to discuss this paper formally, since I have not had a chance to study it in advance. But I am glad for the opportunity to say that this was a most excellent and interesting presentation.

As Dr. Hughes has pointed out, we have done extensive investigations along similar lines in Iowa City and I can only confirm everything that Dr. Thorpe has had to say. We have also been able to study the periphery of lens and vitreous and, in some cases, the ciliary body by means of slitlamp gonioscopy. I am going to present tomorrow some of our observations during accommodation and the similarity of some of our slides and those shown by Dr. Thorpe will be apparent.

I was much interested in some of the details of Dr. Thorpe's presentation. You may recall, for example, a relucent line which Dr. Thorpe has seen in patients after intracapsular cataract extraction. He has called this line the ligamentum hyaloideocapsulare of Wiegert. We have observed this same line and I have considered it, for want of a better explanation, to be the collapsed zonule. Dr. Thorpe's thought that it is the ligamentum hyaloideocapsulare has much to recommend it. It is well seen in the intact eye and I shall point it out again tomorrow on some of our slides.

Another point which I wish to bring out has to do with the flattened appearance of the central part of the lens. Dr. Thorpe explains this on an optical basis and he may well be right. We have noted this angulation and have seen it increase during accommodation.

I wonder whether this might after all not be an actual angulation rather than a purely optical effect as assumed by Dr. Thorpe.

If his paper does what it should do—namely, inspire us to use this methd of examination more frequently—it will have fulfilled an important task. In any event, I am sure that everybody here has enjoyed this paper as greatly as I have.

THE PHOTORECEPTOR PROCESS IN VISION*

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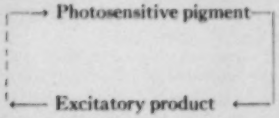
PART I. THE CHEMISTRY OF VISUAL EXCITATION

In this paper I should like to review briefly the present status of the chemistry of visual excitation, specifically in vertebrates; and then to discuss connections between these processes and visual physiology and pathology.

Light initiates a nervous excitation in the outer segments of the rods and cones which, transmitted from one neurone to another to centers in the brain, ends in exciting visual sensations. To achieve this result probably the whole apparatus must be thrown into activity; yet all of it waits upon, and to a degree retains the impress of the primary processes of excitation in the receptor cells.

The general arrangement of these processes is clear beforehand. Light, to have any effect, chemical or physical, must be absorbed. The rods and cones must contain substances which absorb visible light—hence pigments—and are changed thereby so as to yield a nervous excitation. The photosensitive pigments must be continuously restored, or vision would cease soon after a light went on. The excitatory state must also be rapidly removed, or vision would continue long after a light went off. It would aid the economy of such a system if the latter reactions were coupled so as to complete a cycle, but this, though an advantage, is not an essential condition. All photoreceptor processes therefore are arranged as follows:



This is not only the basic arrangement for photoreception, but generalized to include other stimuli than light it must also be the form of all neural excitation. Every irritable tissue must contain similar arrangements for reacting with the stimulus, for removing its effects, and for restoring the original system. The same fundamental pattern of reactions is encountered therefore at every level of the visual pathways; and the entire process of visual excitation from rods and cones to cerebral cortex may be conceived as a chain of such systems. The peculiar importance of the photoreceptor systems rests, not on their intrinsic form, but on their unique sensitivity to light and their initial position in the chain, by virtue of which certain of their properties are imposed on the entire visual response.

Four visual pigments are known: rhodopsin and porphyropsin in rods, and iodopsin and cyanopsin in cones. All of them are built upon a common plan. They are all carotenoid-proteins, proteins bearing carotenoid chromophores, to which they owe their color and sensitivity to light.

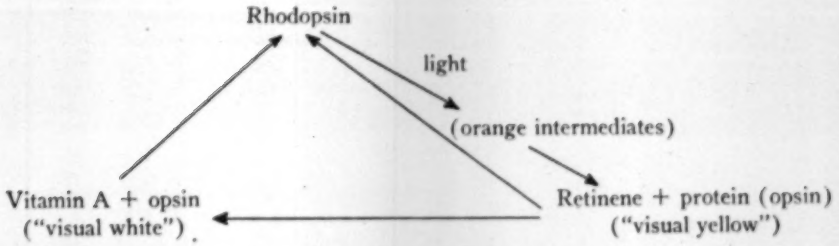
I shall describe the rhodopsin system in some detail, since it provides the model for all the others. Once this system is understood, the others emerge as simple variants upon this common theme.

1. RHODOPSIN

Franz Boll discovered the red pigment rhodopsin in the rods of frogs in 1876. It is characteristic of the rods of marine fishes and land vertebrates (Wald, 1945-46). There is no evidence that it is ever found in cones.

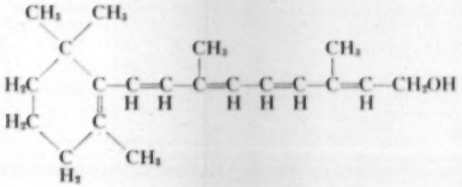
Some years ago rhodopsin was shown to participate in a cycle which seemed to possess the following skeletal form (Wald, 1935-36):

* From the Biological Laboratories of Harvard University. The investigations from our laboratory reviewed in this paper were supported in part by the Rockefeller Foundation, the Office of Naval Research, and the Public Health Service.

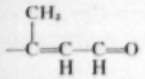


Rhodopsin bleaches in the light over orange intermediates to a mixture of the yellow carotenoid retinene and the colorless protein, opsin (fig. 1). The retinene is then converted to colorless vitamin A. Two processes resynthesize rhodopsin, on the one hand from retinene and opsin, on the other from vitamin A and opsin.

Morton has shown that retinene is vitamin A aldehyde (Ball, Goodwin, and Morton, 1948):



Vitamin A, $C_{19}H_{29}OH$



Retinene, $C_{19}H_{27}O$

The retinene formed by the bleaching of rhodopsin is reduced to vitamin A by the enzyme, alcohol dehydrogenase, working together with the coenzyme, cozymase or DPN. This process is readily carried out in free solution (fig. 2). It involves only the transfer of two hydrogen atoms from reduced cozymase to the aldehyde group of retinene, reducing it to the alcohol group of vitamin A (Wald and Hubbard, 1948-49; Wald, 1950; Bliss, 1951):

$$\underset{\text{retinene}}{C_{19}H_{27}CHO} + \text{DPN-H} + H^+ \xrightarrow[\longleftarrow]{\text{alcohol dehydrogenase}} \underset{\text{vitamin A}}{C_{19}H_{27}CH_2OH} + \text{DPN}^+$$

DPN introduces a second vitamin into the chemistry of vision. Its active principle is nicotinamide, the antipellagra factor of the vitamin-B complex. In the retina it is in the curious position of helping to regenerate vitamin A.

This completes the degradative processes in vision. Rhodopsin, having been bleached by light to a mixture of retinene and opsin, the retinene is reduced to vitamin A. The problem now is to go back. Kühne (1879) already recognized this to be two problems rather than one. He described a resynthesis of rhodopsin from yellow precursors (anagenesis), that was relatively rapid and occurred not only in the intact eye but in the isolated retina and even slightly in solution. In addition there occurred a relatively slow synthesis of rhodopsin from colorless precursors (neogenesis), which Kühne could observe only in the intact eye, and which seemed to require the co-operation of the

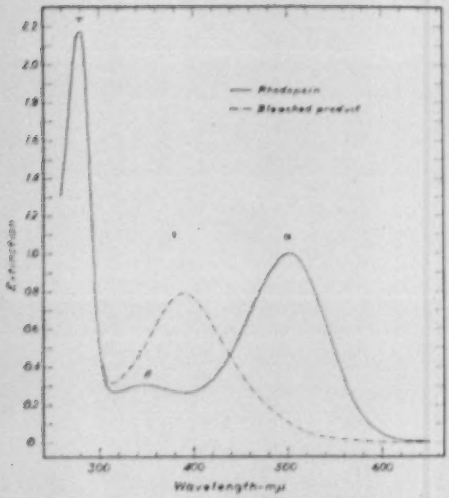


Fig. 1 (Wald). Absorption spectra of bullfrog rhodopsin and of the product of its bleaching in aqueous digitonin solution (pH 5.55). Rhodopsin possesses three absorption maxima: the α-band, mainly responsible for the spectral sensitivity of rod vision; the β-band, which, like α-, belongs to the prosthetic group; and the γ-band, due to the protein opsin. On bleaching, the α- and β-bands are replaced by the retinene band at about 385 mμ; the opsin band remains unchanged (from Wald, 1949).

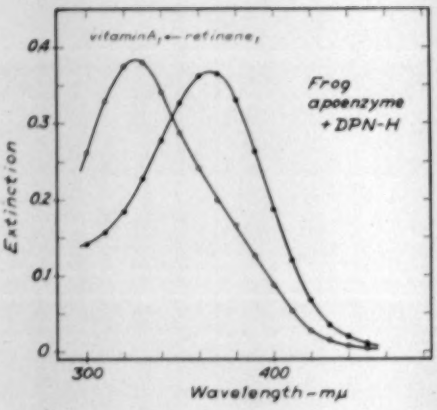


Fig. 2 (Wald). The reduction of retinene to vitamin A. Retinene was mixed in digitonin solution with the enzyme, alcohol dehydrogenase, extracted from frog retinas, and with reduced cozymase (DPN-H). A control mixture was also prepared, which differed only in that the enzyme had been kept at 100°C. for one-half minute. Both mixtures were incubated, then extracted with hexane. The absorption spectra of the hexane extracts are shown. That from the control mixture (solid circles) is the spectrum of unaltered retinene; that from the mixture containing active enzyme (open circles) shows complete conversion to vitamin A (from Wald, 1950).

pigment epithelium. We can identify these two processes now with the synthesis of rhodopsin from retinene and opsin, and from vitamin A and opsin.

The synthesis of rhodopsin from retinene and opsin is a spontaneous reaction. It requires neither an enzyme nor—as do most syntheses—an external source of energy. One has only to bring a mixture of these two substances into the dark to form rhodopsin. This is therefore, like all spontaneous reactions, an energy-yielding process. It is the *bleaching* of rhodopsin that requires energy, ordinarily provided by light (Wald and Brown, 1950).

The synthesis of rhodopsin from vitamin A and opsin is more complex. The equilibrium between vitamin A and retinene lies far over toward the side of reduction—toward vitamin A. In the dark, however, opsin "traps" retinene, removing it to form rhodopsin, so displacing the equilibrium in the oxidative direction. The basic mechanism of rhodopsin synthesis, therefore, is the energy-demanding oxidation of vitamin A to retinene, coupled with the energy-yielding condensation of retinene and opsin to form rhodopsin (Wald and Hubbard, 1950; Hubbard and Wald, 1951).

The rhodopsin system therefore has the form shown in Figure 3. Rhodopsin is converted by light to the orange-red intermediate, lumi-rhodopsin. At temperatures above −20°C. this goes on to form meta-rhodopsin; and with access to water, meta-rhodopsin yields retinene and opsin (Wald, Durell, and St. George, 1950). The retinene is then reduced to vitamin A. In the dark, the spontaneous combination of retinene and opsin to form rhodopsin promotes the oxidation of vitamin A to retinene. This process is aided by the influx of new vitamin A from the pigment epithelium, which obtains it from

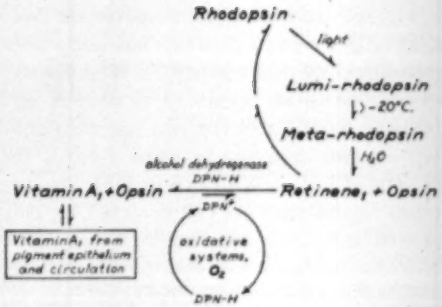


Fig. 3 (Wald). Diagram of the rhodopsin system (from Hubbard and Wald, 1951).

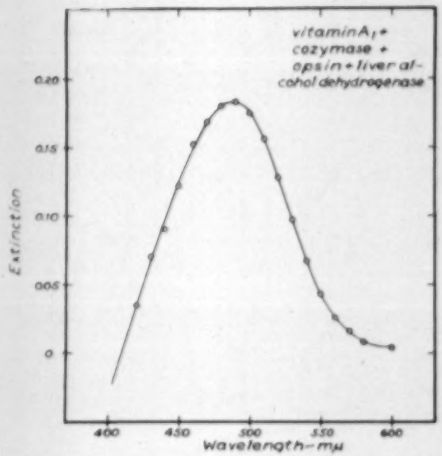


Fig. 4 (Wald). Synthesis of rhodopsin by a mixture of four substances in aqueous digitonin solution: fish liver vitamin A, cozymase, frog opsin, and horse liver alcohol dehydrogenase. This mixture was incubated in the dark, and its absorption spectrum measured. It was then bleached in the light, and its spectrum re-measured. The difference between these spectra, shown in the figure, resembles the difference spectrum of rhodopsin. Actually this experiment was done before we realized the role of *cis-trans* isomers of vitamin A in this process. Our fish liver vitamin A contained mainly the iso-*a* isomer, in addition to inactive forms, and what was synthesized here was mainly iso-rhodopsin. Had we begun with the neo-*b* isomer of vitamin A, we would have obtained pure rhodopsin (from Hubbard and Wald, 1951).

the blood circulation; by the provision of DPN, the oxidant of vitamin A; and by respiratory enzymes, which keep cozymase oxidized. All these factors acting in concert sweep the system back toward rhodopsin (Hubbard and Wald, 1951).

Judging from Figure 3, it should be possible to assemble the rhodopsin system by mixing four substances in solution: vitamin A, opsin, alcohol dehydrogenase, and DPN. Such mixtures have been assembled, using vitamin A from fish liver oil, crystalline alcohol dehydrogenase from horse livers, and DPN from yeast. The only component that needs to be obtained from the retina, and indeed from the outer segments of the rods, is opsin. Such a mixture, placed in the dark, forms rhodopsin (fig. 4). Brought into the light, it bleaches; and replaced in the dark, it synthesizes more rhodopsin. This mixture of substances performs in solution all the reactions of the rhodopsin system (Hubbard and Wald, 1951).

We found, however, that in making up this mixture, not all vitamin A is effective. Rhodopsinlike pigments were synthesized from liver oil vitamin A, but almost none from ordinary crystalline or synthetic vitamin A. This observation led to a further development.

Vitamin A, like other carotenoids, exists in a number of different molecular shapes, cis-trans isomers of one another (Zechmeister, 1944, 1954; Robeson and Baxter, 1947). The usual crystalline or synthetic vitamin A is primarily the all-*trans* isomer (fig. 5). It is this that we had found ineffective in rhodopsin synthesis. Apparently rhodopsin requires for its formation one of the *cis* isomers of vitamin A (Hubbard and Wald, 1952-53).

According to theory, only two of the four side-chain double bonds of vitamin A should have been capable of forming stable *cis*-linkages, those marked 9 and 13 in Figure 5. At the other double bonds, a *cis* linkage encounters serious steric hindrance, and the molecule must be twisted out of coplanarity. This interferes with resonance, and should consequently lead to a lowered stability (Pauling, 1939, 1949). Only four geometric

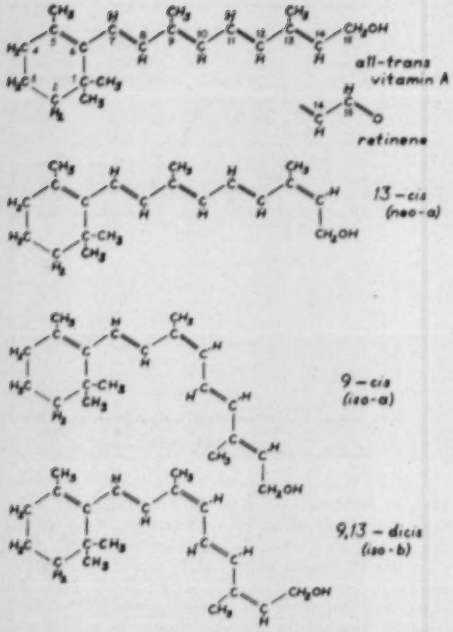


Fig. 5 (Wald). Unhindered geometric isomers of vitamin A. This molecule can assume the *cis* configuration only at double bonds 9 and 13 without encountering serious steric hindrance. At the other double bonds, groups come into conflict, and the *cis* configuration not only bends but twists the molecule (modified from Hubbard and Wald, 1952-53).

isomers of vitamin A or retinene were therefore expected: all-*trans*, 9-*cis*, 13-*cis*, and 9,13-*dicis* (fig. 5).

Five cis-trans isomers of retinene, however, have been isolated in crystalline condition: the all-*trans* isomer, originally prepared by Morton et al.; neoretinenes *a* and *b*, first isolated in our laboratory, and the latter since prepared in purer condition by Dieterle and Robeson (1954); and isoretinenes *a* and *b*, prepared by the Organic Research Laboratory of Distillation Products Industries in Rochester, New York (Hubbard and Wald, 1952-53; Hubbard, Gregerman and Wald, 1952-53).

All the unhindered isomers have now been identified. Neo-*a* is 13-*cis;* iso-*a*, 9-*cis;* and iso-*b*, 9,13-*dicis*. It is, however, the fifth isomer, neo-*b* that makes rhodopsin. This must therefore be a hindered form.

The existence of stable hindered *cis* linkages in this type of molecule has lately been established by direct synthesis. The properties of the neo-*b* isomer of vitamin A and retinene show that it contains one such linkage. It must therefore be either 7-*cis* or 11-*cis* (fig. 6). Dr. William Oroshnik has recently synthesized 11-*cis* vitamin A. This proved not to be the neo-*b* isomer. The latter therefore must be 7-*cis* (Wald, Brown, Hubbard, and Oroshnik, 1955).

This is the most hindered, and was expected to be the least likely, of all possible isomers of vitamin A. It is a surprise to find that it exists at all. Yet this is the form on which we depend in both rod and cone vision.

The synthesis of rhodopsin proceeds in two stages. First, vitamin A is oxidized to retinene; then retinene couples with opsin. The first process is relatively indifferent to isomeric configuration. It is the coupling of retinene with opsin that is isomer-specific.

On incubation with opsin in the dark, neo-*b* retinene yields rhodopsin (λ_{max} 500 mμ), indistinguishable from that extracted

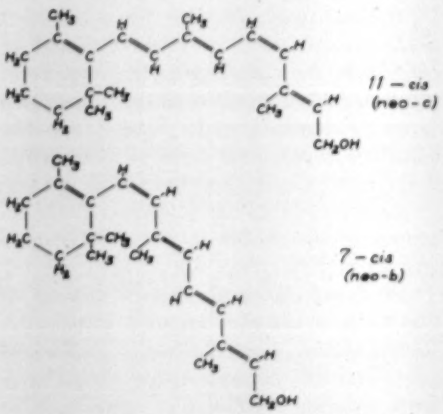


Fig. 6 (Wald). Hindered geometric isomers of vitamin A. These are the forms thought until recently to be so improbable as not to exist in appreciable quantities. Since the 11-*cis* isomer (neo-*c*) is not neo-*b*, the precursor of rhodopsin and iodopsin, the latter must be 7-*cis*. This is the most hindered configuration that vitamin A can assume (from Wald, Brown, Hubbard, and Oroshnik, 1955).

from the dark adapted retina. On similar treatment, iso-*a* retinene yields a very similar, light-sensitive pigment, with its λ_{max} displaced to 487 mμ. This is called isorhodopsin. The remaining isomers are inactive (fig. 7).

Which isomer of retinene emerges when rhodopsin is bleached? This proved to be an inactive form, indeed all-*trans* retinene. It must be isomerized to the active isomer, neo-*b*, before it can re-synthesize rhodopsin. A cycle of *cis-trans* isomerization is therefore an intrinsic part of the rhodopsin system.

A single passage through this cycle is shown in Figure 8. On the left, a mixture of neo-*b* retinene and cattle opsin in aqueous solution, incubated in the dark, forms rhodopsin. On the right, the rhodopsin formed in this way is bleached to a mixture of all-*trans* retinene and opsin. It will be noted that the extinction of retinene which emerges on the right is much higher than that which entered on the left. That is because the specific extinction of all-*trans* retinene is higher than that of the neo-*b* isomer.

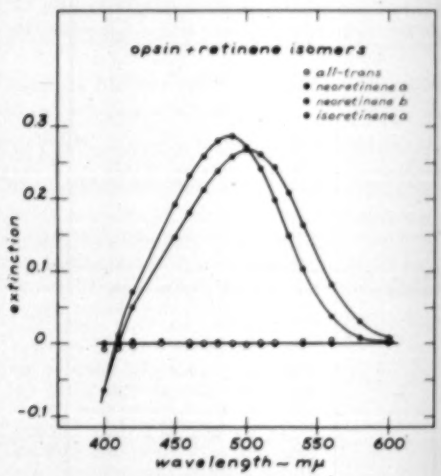


Fig. 7 (Wald). The products of incubating various geometric isomers of retinene with cattle opsin. Difference spectra are shown—differences in the absorption spectra before and after bleaching in the presence of hydroxylamine. All-*trans* and neo-*a* retinene yield no light-sensitive pigment. Neo-*b* retinene yields rhodopsin, iso-*a* retinene iso-rhodopsin. Iso-*b* retinene, though itself inactive, isomerizes preferentially to iso-*a*, which yields iso-rhodopsin (from Hubbard and Wald, 1952-53).

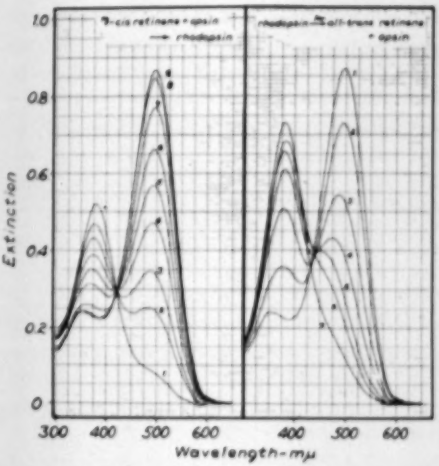
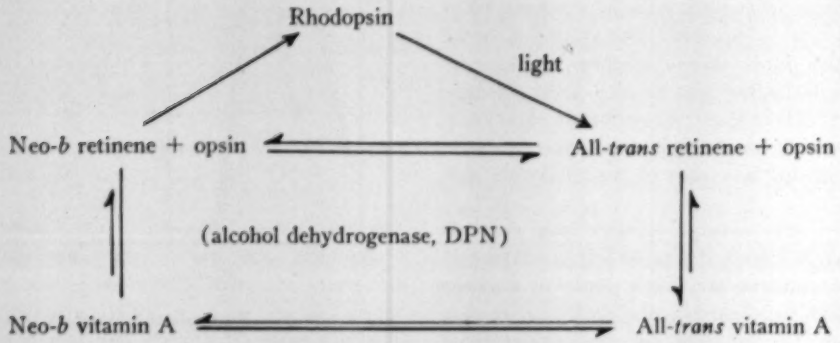


Fig. 8 (Wald). Synthesis and bleaching of rhodopsin in solution (22.5°C., pH 7.0). *Left:* a mixture of neo-*b* retinene and cattle opsin was incubated in the dark, and absorption spectra recorded periodically, (1) at 0.3 minutes, (2) at 2.5 minutes, (3) five minutes, (4) 10 minutes, (5) 18 minutes, (6) 30 minutes, (7) 60 minutes, (8) 120 minutes, and (9) at 180 minutes. The absorption band of neo-*b* retinene (λ_{max} 380 mμ) falls regularly, while that of rhodopsin (λ_{max} 498 mμ) rises. *Right:* the rhodopsin formed at the left (1) is exposed to light of wavelengths >550 mμ for various intervals, and the spectrum is recorded immediately after each exposure. The total irradiations are: (2) five seconds, (3) 10 seconds, (4) 15 seconds, (5) 30 seconds, and (6) 120 seconds. The residue was exposed for 45 seconds longer to light of wavelengths >440 mμ (7). (From Wald and Brown, 1955.)

The all-*trans* retinene which results from bleaching rhodopsin can be isomerized to an equilibrium mixture of *cis* and *trans* isomers by simple exposure to light. This is a second photochemical process in the rhodopsin system. The eye tissues also contain enzyme, an isomerase, which catalyzes specifically interconversions of all-*trans* and neo-*b* retinene, and which also is light-sensitive (Hubbard, 1955). In the eye, before such isomerizations can be completed, much of the all-*trans* retinene is reduced to all-*trans* vitamin A; and this in turn must be isomerized

to neo-*b* vitamin A before it can be used again in vision.

The rhodopsin system can therefore be formulated as follows (Hubbard and Wald, 1952-53):



A further reaction is involved in this system, though its function is still obscure. This is the esterification of vitamin A. Vitamin A is expected to emerge from the bleaching of rhodopsin as the free alcohol; yet the great bulk of the vitamin A we find in the retina is in the form of an ester. Recently a cell-free enzyme system has been prepared from cattle retinas that esterifies vitamin A *in vitro* (Krinsky, 1955). Superficially this process has the appearance of an extra step, a gratuitous reaction that needs to be reversed for the vitamin A to go back into the visual processes. I feel sure that this is a superficial view, and that as we understand better the workings of the retina, this process also will be found to play an important role.

2. Porphyropsin

The rods of vertebrates which live in, or better, spawn in, fresh water—freshwater fishes, lampreys, and certain larval and adult amphibia—characteristically contain in place of rhodopsin a purple light-sensitive pigment, called porphyropsin (Wald, 1945-46). Its λ_{max} in aqueous solution is 522 ± 2 mμ

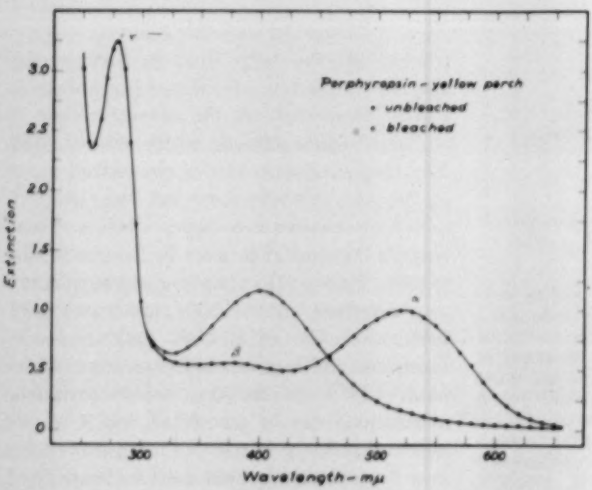
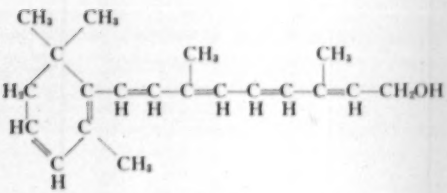
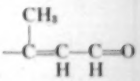


Fig. 9 (Wald). Absorption spectra of porphyropsin and of the product of its bleaching (pH 7.0), from the freshwater yellow perch, *Perca flavescens*. This preparation was extracted with two-percent digitonin from a suspension of the outer segments of the retinal receptors, which had been previously hardened with alum, and pre-extracted with water and with petroleum ether. Porphyropsin, like rhodopsin, possesses three absorption bands: the α-band at about 522 mμ, the β-band at about 377 mμ, and the γ-band (opsin) at about 280 mμ. On bleaching, the α- and β-bands are replaced by the absorption band of retinene, at about 400 mμ (Wald, Brown, and Smith, unpublished observations).

(fig. 9). On bleaching, it yields a mixture of opsin and a new retinene, called retinene$_2$, and this in turn is reduced to a new vitamin A, called vitamin A$_2$. It was the analysis of this visual system that led to the discovery of these carotenoids (Wald, 1937 (a); 1938-39). After many years of confusion, the structures of these substances were established by total synthesis, in the laboratory of E. R. H. Jones in Manchester (Farrar et al., 1952). They differ from vitamin A and retinene only in possessing an added double bond in the ring:



Vitamin A$_2$, $C_{20}H_{27}OH$



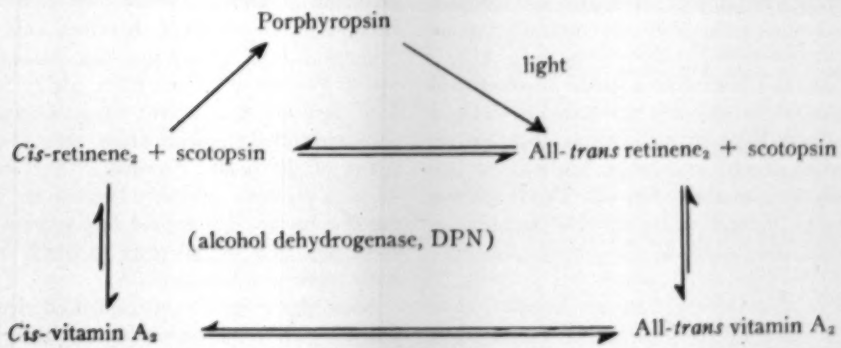
Retinene$_2$, $C_{20}H_{26}O$

The properties of the porphyropsin system are in general precisely parallel with those of the rhodopsin system. Alcohol dehydrogenase and DPN catalyze the same equilibrium between retinene$_2$ and vitamin A$_2$ as between retinene and vitamin A (Wald, 1950). The bleaching of porphyropsin yields an inactive form of retinene$_2$, apparently the all-*trans* isomer.

We have not yet come as far with the geometric isomers of retinene$_2$ as with those of retinene. We have, however, isolated two *cis* forms of retinene$_2$, neither crystalline nor pure, one of which, incubated in the dark with opsin, yields porphyropsin, indistinguishable from that extracted from a dark-adapted freshwater fish retina; while the other, treated similarly, yields a comparable pigment, isoporphyropsin, with λ_{max} 507 mμ (fig. 10) (Wald, Brown, and Smith, unpublished observations, reviewed in Wald, 1953, 1954).

In performing such syntheses it makes little difference whether one uses opsin from a freshwater fish, a frog, or cattle. All these opsins yield typical porphyropsins. The pigments obtained with cattle opsin lie at slightly shorter wavelengths than those obtained with frog opsin; cattle "porphyropsin" lies at λ_{max} 517 mμ, and cattle "isoporphyropsin" at λ_{max} 501 mμ. It should be noted, however, that cattle and frog *rhodopsins* display similar differences; the former has λ_{max} 498 mμ, the latter λ_{max} 502 mμ.

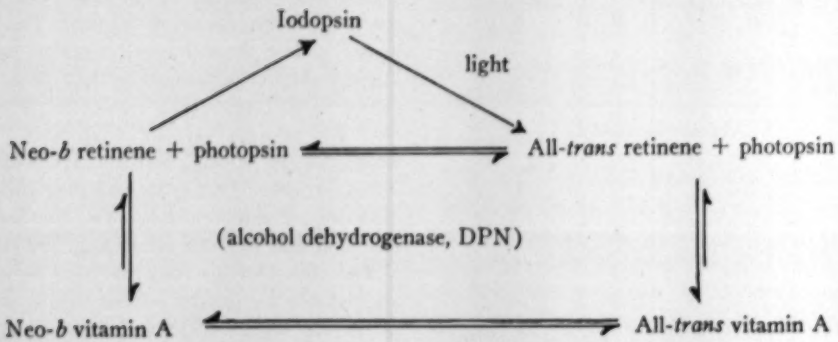
The opsins of the rods that have been examined are so closely related that it seems proper to regard them as belonging to the same family, the *scotopsins*. The rhodopsin and porphyropsin systems therefore share entirely the same proteins. It is only their carotenoids which differ, and that only by the added double bond in the ring. The porphyropsin system can be formulated:



3. Iodopsin

The first light-sensitive pigment of cone vision was extracted from the chicken retina in 1937. It is a violet pigment (λ_{max} 562 mμ) called iodopsin. The chicken retina contains a few rods among a large predominance of cones, and yielded a mixture of iodopsin and rhodopsin (Wald, 1937 (b)).

The carotenoids of the iodopsin system are identical with those of the rhodopsin system, even to *cis-trans* configuration. Only the opsin is different. Having designated the opsins of the rods as scotopsins, we may call those of the cones photopsins. The replacement of scotopsin by photopsin changes the rhodopsin to the iodopsin system (Wald, Brown, and Smith, 1954-55):



From the light-adapted chicken retina one can extract a colorless, carotenoid-free mixture of the proteins of rod and cone vision, scotopsin and photopsin. On incubating this, or a wholly bleached extract of chicken retinas, in the dark with neoretinene *b*, one obtains a mixture of rhodopsin and iodopsin indistinguishable from that extracted from the dark-adapted chicken retina (fig. 11).

Just as isoretinene *a* yields iso-rhodopsin when incubated with scotopsin, it yields a similarly displaced pigment, iso-iodopsin, on incubation with photopsin. The λ_{max} of iso-iodopsin is at about 510 mμ. The remaining isomers of retinene are inactive (fig. 12).

4. Cyanopsin

The visual systems so far described have been made by coupling either rod or cone opsin with either retinene$_1$ or retinene$_2$. Scotopsin combines with retinene$_1$ to yield rhodopsin, or with retinene$_2$ to yield porphyropsin. Photopsin combines with retinene$_1$ to yield iodopsin. Clearly a fourth combination is possible: photopsin with retinene$_2$.

We have recently made this in the laboratory. The result is a *blue* photosensitive pigment called cyanopsin. Its maximal absorption is in the orange-red, at about 620 mμ. We have also made iso-cyanopsin; its λ max. is 575 mμ (Wald, Brown, and Smith, 1953).

Always heretofore our knowledge of a visual pigment had developed in the sequence: recognition, extraction, analysis, synthesis. With cyanopsin this history was reversed. We had synthesized a pigment in solution which had never been identified in a retina. Had it a place in vision?

Where would one look? Obviously in retinas which provide the two necessary ingredients: cones, and hence photopsin; and vitamin A$_2$. One might therefore look for cyanopsin in a freshwater fish possessing cones. We knew of one other place. Some time ago we had attempted to extract a photosensitive pigment from the all-cone retina of the turtle, *Pseudemys*. We found no such pigment, probably because we used too few turtles; but we did find vitamin A$_2$. Here then is a second place in which cones come together with vitamin A$_2$.

Some years ago Granit measured electrophysiologically the spectral sensitivity of

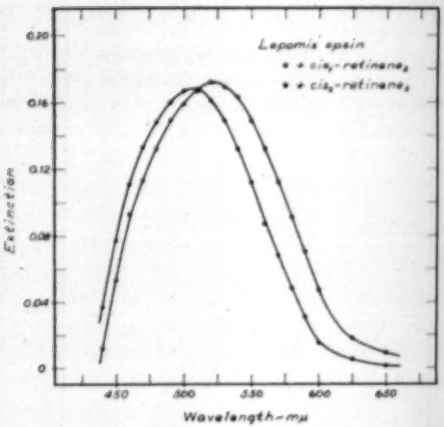


Fig. 10 (Wald). Synthesis of prophyropsin and iso-porphyropsin. Two different preparations, containing partially purified *cis* isomers of retinene$_2$, were incubated with opsin from the sunfish, *Lepomis*. The difference spectra of the products are shown, measured in the presence of hydroxylamine. The *cis*$_1$ isomer yields porphyropsin (λ_{max} 522 mμ), the *cis*$_2$ isomer iso-porphyropsin (λ_{max} 507 mμ). (Wald, Brown, and Smith, unpublished observations).

cone vision in a freshwater fish, the tench, and in the European tortoise, *Testudo graeca* (Granit 1941 a, b). His measurements are shown as the points in Fig. 13; the line is the visible absorption band of cyanopsin. I think there is little doubt that cyanopsin is the pigment of cone vision in these animals.

5. Recapitulation

This phase of the chemistry of visual excitation ends on a very simple note. Each of the visual systems we have studied involves the interaction of four substances: a rod or cone opsin; the enzyme, alcohol dehydrogenase; the coenzyme, cozymase; and a specific *cis* isomer of vitamin A_1 or A_2. By incubating in the dark rod or cone opsin with the proper isomer of retinene$_1$ or retinene$_2$ one obtains as products:

	λ_{max}(mμ)
Rod opsin + retinene$_1$ → rhodopsin	500
Rod opsin + retinene$_2$ → porphyropsin	522
Cone opsin + retinene$_1$ → iodopsin	562
Cone opsin + retinene$_2$ → cyanopsin	620

In addition we have the four iso-pigments, whose carotenoid moieties are stereoisomeric with those listed above. None of the iso-pigments has yet been found in a retina. All of them must for the present be regarded as artefacts. How does the retina avoid forming them? It seems from preliminary measurements in our laboratory that the iso-*a* isomer of vitamin A, the

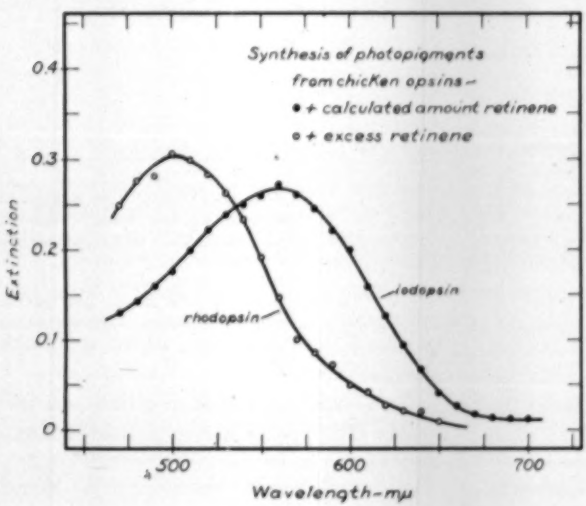


Fig. 11 (Wald). Successive syntheses of iodopsin and rhodopsin in solution. An extract of chicken retinas was wholly bleached with an orange non-isomerizing light to a mixture of all-*trans* retinene and rod and cone opsins. To this mixture just enough neo-*b* retinene was added to regenerate iodopsin alone. This amount had been determined by preliminary trial. Iodopsin forms so much more rapidly than rhodopsin that its synthesis is complete when that of rhodopsin has scarcely begun (cf fig. 19). The absorption spectrum of the product, formed within a few minutes in the dark, is shown with solid circles. Then a small excess of neo-*b* retinene was added, and the mixture was reincubated in the dark for 30 minutes. This yielded rhodopsin (open circles) (from Wald, Brown, and Smith, (1954-55).

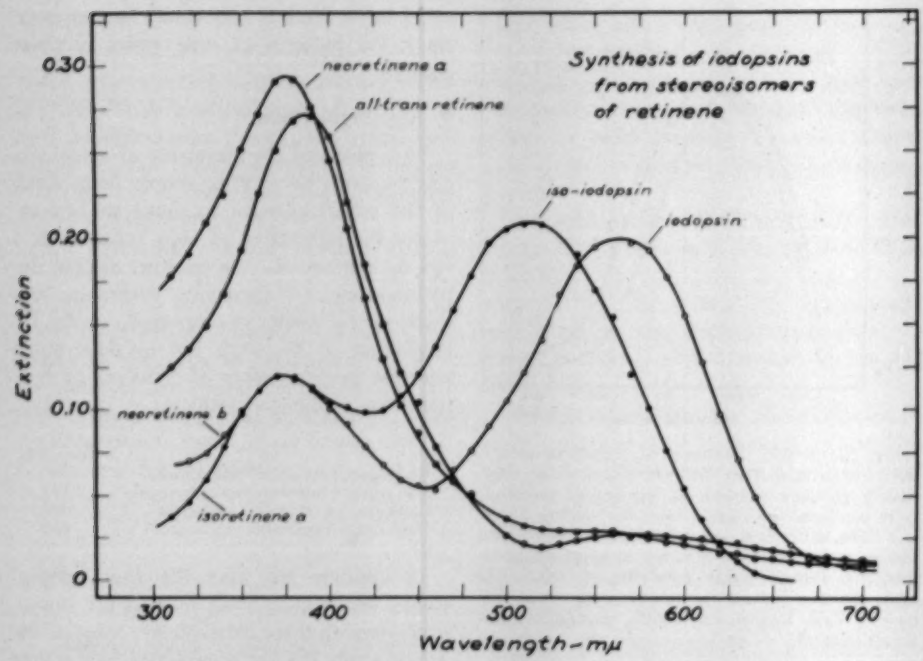


Fig. 12 (Wald). Synthesis of iodopsin and iso-iodopsin. In a chicken retinal extract, the iodopsin alone was bleached with deep red light to a mixture of all-*trans* retinene and photopsin. This product was incubated in the dark with four geometric isomers of retinene. The absorption spectra were then measured against the red-bleached solution as blank. All-*trans* and neo-*a* retinene had remained almost as added. Neo-*b* retinene had formed a large amount of iodopsin (λ_{max} 562 mμ), iso-*a* retinene iso-iodopsin (λ_{max} 510 mμ). Both photosensitive pigments are accompanied by residues of unchanged retinene, primarily responsible for the absorption bands at about 370 mμ (from Wald, Brown, and Smith, 1954-55).

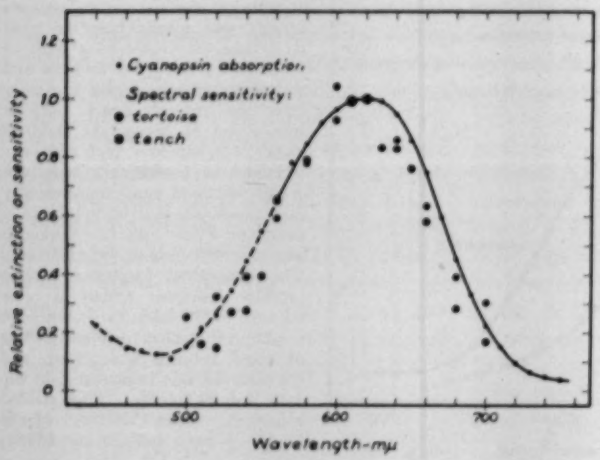


Fig. 13 (Wald). The absorption spectrum of cyanopsin, compared with Granit's electrophysiologic measurements of the spectral sensitivity of cone vision in a fresh-water fish, the tench, and in the European tortoise, *Testudo graeca* (from Wald, Brown, and Smith, 1953).

precursor of iso-rhodopsin and iso-iodopsin, is present in considerable quantity in liver oils, and probably also in blood. It would seem to be readily available to all the tissues; whereas the highly hindered neo-*b* isomer has not yet been identified anywhere but in the retina, and probably always requires special dispensations for its formation. Yet it is the latter with which we see.

6. The visual pigments as proteins

To this point we have discussed the visual pigments mainly from the point of view of their carotenoid components. Their properties depend greatly, however, also upon the opsins. Though their color and sensitivity to light are mediated principally through the carotenoid prosthetic groups, almost everything else derives from their character as proteins.

Rhodopsin has been studied most in this regard. Cattle rhodopsin has a molecular weight of about 40,000 grams per mole (Hubbard, 1953-54). Each molecule contains a single unit of retinene (Wald, 1953 b; Hubbard, 1953-54). The extinction of a molar solution, measured in a layer one cm. in depth, is 40,600 (Wald and Brown, 1953-54). The isolectric point of frog rhodopsin is at pH 4.47, and goes to pH 4.57 on bleaching (Broda and Victor, 1940).

The synthesis of rhodopsin from retinene and opsin requires the presence of free sulfhydryl (-SH) groups on opsin. The synthesis is unaffected by relatively high concentrations of monoiodoacetate (0.03 M); but it is blocked completely by very low concentrations of the more powerful and specific sulfhydryl poison, p-chloromercuribenzoate (7×10^{-6} M). Conversely, the bleaching of rhodopsin liberates sulfhydryl groups, in the proportions 2-3 -SH groups per retinene. This is true equally of rhodopsins from cattle, frogs, and squid (Wald and Brown, 1951-52; 1953-54). It is not yet clear whether in rhodopsin the -SH groups participate directly in binding retinene to opsin, or are merely unavailable to sulfhydryl reagents until exposed by bleaching.

It is significant in this connection that the bleaching and resynthesis of rhodopsin resemble in many ways a reversible protein denaturation. This was first suggested by Mirsky (1936). Rhodopsin is bleached as well by heat as by light, or by combinations of heat and light, always to essentially the same products (St. George, 1951-52). Bleaching is reported to shift the isolectric point slightly in the alkaline direction Broda and Victor, 1940); denaturation has the same effect on a number of proteins. Rhodopsin, though converted to colored intermediates by light, requires the presence of water to bleach (Wald, Durell, and St. George, 1950). The need for water in protein denaturation has been familiar since the work of Chick and Martin (1910). The exposure of new sulfhydryl groups on bleaching is another such property; this is one of the characteristic signs of protein denaturation.

All of this means that the bleaching of rhodopsin, in addition to splitting off carotenoid, works profound changes in the configuration of opsin, which in turn must be reversed when rhodopsin is synthesized. As we come to approach more closely the question, how the attack of light upon rhodopsin results in excitation, the changes in opsin may well prove more significant than the more visible changes which involve the prosthetic group.

Part II. Physiologic correlations

Every physiologic function, normal and pathologic has its roots in biochemistry; conversely every facet of biochemistry finds expression in the properties and behavior of the organism. In a sense the organism is a macroscopic representation of certain of its component molecules; and one of the principal tasks of physiology is to learn to read its features in their features.

This is nowhere plainer than in vision. The reactions initiated by light in the rods and cones introduce a long train of nervous

and synaptic processes which end in overt acts and visual sensations. We have described the primary events in some detail. The visual apparatus as a whole is largely concerned with conducting information about them. For this reason many of the basic properties of vision reflect simply and directly the properties of retinal molecules.

It is of the highest importance to explore these relationships. They will not prove exhaustive; for there is much more in vision than photochemistry, or indeed than any of the peripheral processes we can measure. Yet it is important to learn how far we can come with these, if only to know that we must seek elsewhere for what remains.

1. Absorption spectra and spectral sensitivity: The Purkinje phenomenon

The rise and fall of visual sensitivity throughout the spectrum is governed in the first instance by the capacity of the visual pigments to absorb light of various wavelengths, that is, by their absorption spectra. When properly corrected, the spectral sensitivity should correspond closely with the absorption spectra of the visual pigments.

For such a comparison, the spectral sensitivity must be corrected for distortions caused by colored ocular structures—in the human eye principally the yellow lens and macula lutea; and similar structures in the eyes of other animals. The spectral sensitivity also should be quantized. What is measured generally is the relative energy at each wavelength needed to evoke a constant response. The reciprocal of this is the relative sensitivity; and this divided by the wavelength is the sensitivity in terms of relative numbers of incident quanta. This is the form in which spectral sensitivity data can best be employed for the present purpose.

The spectra of the visual pigments should be stated in terms of percentage absorption rather than extinction (cf. Wald, 1937-38). The point of this distinction is that all extinction curves are simple multiples of one another; whereas a percentage absorption curve has a unique shape depending upon the actual value of the absorption. However extinction and percentage absorption are almost exactly proportional to each other up to 10 percent absorption; and depart only slightly from proportionality up to about 20 percent absorption. All known cones and most rods have absorptions well below this value. Extinction therefore runs parallel with absorption for all cones and all but the more densely pigmented rods. In the figures which follow, since we usually do not know the percentage absorption, the absorption spectra of the visual pigments have been plotted in terms of relative extinction. This introduces appreciable distortion only in the comparison with frog rod vision (fig. 15).

We do not possess at present accurate measurements of the spectral sensitivities of rod and cone vision in the chicken. Granit (1942) and Donner (1953) have made such measurements on the closely related pigeon retina. The lens and cornea were removed from the eye and microelectrodes inserted into the retina. The pigeons were either dark adapted one to two hours following the operation, or were light adapted. At each wavelength, the energy was measured needed to evoke a constant electrical response. The reciprocal of the energy—the sensitivity—was quantized by dividing by the wavelength.

Figure 14 shows the comparison between these measurements and the absorption spectra of chicken rhodopsin and iodopsin. The scotopic sensitivity agrees very well with the absorption spectrum of rhodopsin. The photopic sensitivity, however, is displaced about 20 mμ toward the red from the spectrum of iodopsin. This displacement must be caused in large part by the brightly colored oil globules which lie in the cones of chicken and pigeons in the position of color filters (Walls and Judd, 1933; Wald and Zussman, 1938). The displacement seems larger than the color filters of the chicken retina should cause; and may mean that many of the electrophysiologic measurements happened to fall within the "red field" of the

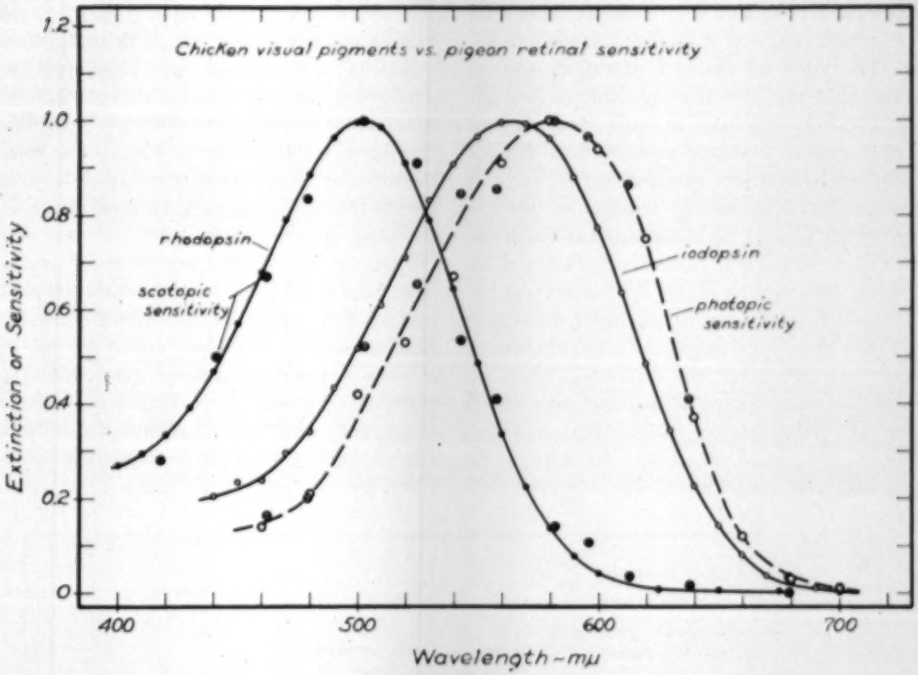


Fig. 14 (Wald). Absorption spectra of chicken rhodopsin and iodopsin, compared with the spectral sensitivities of dark and light adapted pigeons. The latter were measured electrophysiologically, and are plotted in terms of the reciprocals of the numbers of incident quanta needed to evoke a constant electrical response. The scotopic data are from Donner (1953), the photopic data from the same source (barred circles) and from Granit (1942c; open circles). The scotopic sensitivity agrees well with the absorption spectrum of rhodopsin. The photopic sensitivity is displaced about 20 mμ toward the red from the absorption spectrum of iodopsin, owing in large part to the filtering action of the colored oil globules of the pigeon cones (from Wald, Brown, and Smith, 1954-55).

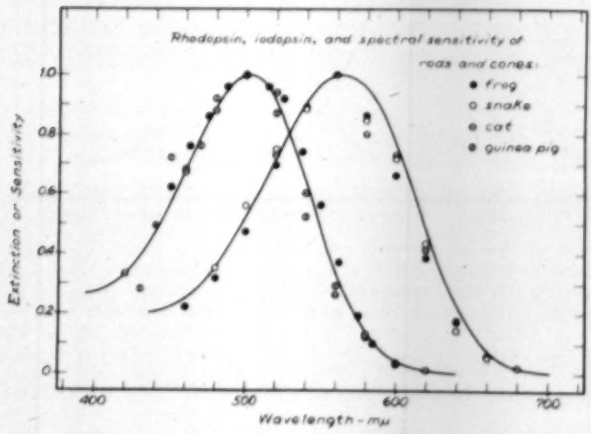


Fig. 15 (Wald). The absorption spectra of chicken rhodopsin and iodopsin, compared with the scotopic and photopic sensitivities of various animals. The lines show the absorption spectra of the visual pigments, the points electrophysiologic measurements of spectral sensitivity (quantized). Scotopic data: frog (Granit, 1947, p. 292); cat (Donner and Granit, 1949); guinea pig (Granit, 1942 b). Photopic measurements: frog (Granit, 1942 a); snake (Granit, 1943 a); cat (Granit, 1943 b) (from Wald, Brown, and Smith, 1954-55).

pigeon retina, the dorsotemporal quadrant in which deep red oil globules predominate.

The shift of spectral sensitivity toward the red as one goes from scotopic to photopic conditions, from rod to cone vision, is the well known Purkinje phenomenon. Except for the distortion just alluded to, this is accurately mimicked in solution by the absorption spectra of rhodopsin and iodopsin.

This comparison gains special force when made with retinas which do not possess obviously colored filtering pigments. In Figure 15 the absorption spectra of chicken rhodopsin and iodopsin are compared with the spectral sensitivities of rod and cone vision in the frog, snake, guinea pig, and cat, measured with electrical procedures by Granit and co-workers. The scotopic data agree very well with the absorption spectrum of rhodopsin. (In the case of the frog, they would agree even better had we plotted the percentage absorption rather than the extinction of rhodopsin.) The photopic sensitivities agree so well with the absorption spectrum of iodopsin that it seems probable that this is the major pigment of cone vision in the frog, snake, and cat.

Figure 15 shows that when colored ocular structures do not intervene, the Purkinje phenomenon emerges directly from the absorption spectra of rhodopsin and iodopsin. In essence it involves nothing more than the transfer of vision from dependence on the absorption spectrum of rhodopsin in dim light to that of iodopsin in bright light.

Figure 16 shows this same comparison

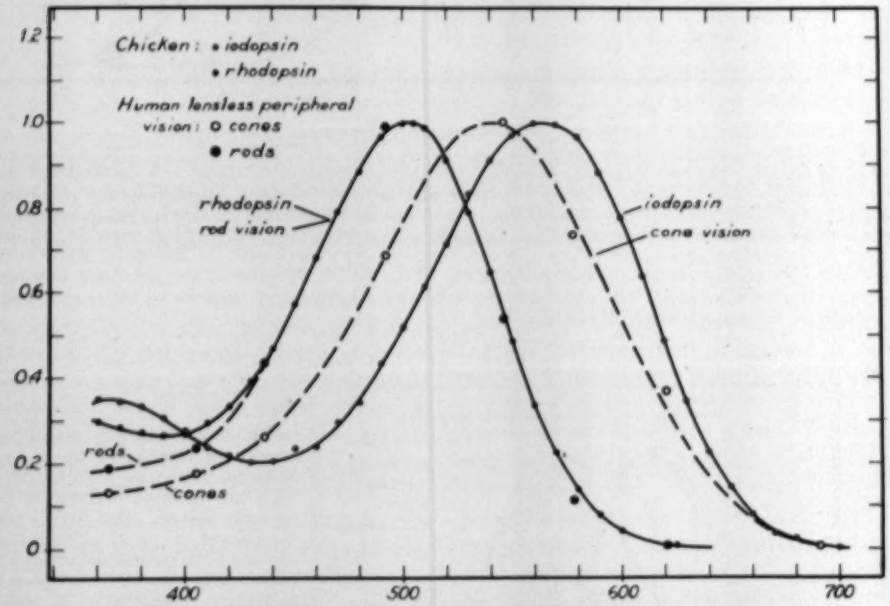


Fig. 16 (Wald). Absorption spectra of chicken rhodopsin and iodopsin compared with the spectral sensitivity of human rod and cone vision. The spectral sensitivity measurements were made in a peripheral field in the aphakic (lensless) eye, to avoid distortions caused by the yellow pigmentations of the lens and macula lutea. They represent as close an approximation to the sensitivities of the naked rods and cones as can be achieved in the living eye (cf. Wald, 1945 b, 1949). The scotopic (rod) sensitivity agrees with the absorption spectrum of rhodopsin over most of its course. The photopic (cone) sensitivity is displaced some 20 mμ toward the blue from the absorption spectrum of iodopsin; it represents the resultant of the spectral sensitivities of at least three groups of cones, concerned with color vision (from Wald, Brown, and Smith, 1954-55).

for the human eye. The spectral sensitivities were measured in the periphery of the aphakic (lensless) eye to avoid distortions otherwise introduced by the yellow pigmentations of the lens and macula lutea (Wald, 1945, 1949). The scotopic sensitivity agrees well with the absorption spectrum of rhodopsin; but the photopic sensitivity is displaced about 20 mμ toward the blue from iodopsin. This is hardly surprising, for the human photopic sensitivity is believed to be a composite function, the resultant of the spectral sensitivities of at least three classes of cone, needed to account for normal trichromatic vision. According to Stiles (1949), these possess maxima at about 440, 550, and 590 mμ (cf. also Auerbach and Wald, 1955). The middle member of the trio could represent iodopsin or a closely related pigment; but in the human cones this must co-operate with at least two other pigments to provide the mechanism of color differentiation.

Finally, in Figure 17, such a comparison is shown for a vitamin A_2 eye, that of a freshwater fish, the tench. The spectral sensitivities, scotopic and photopic, measured electrophysiologically by Granit (1941 b), are shown as large circles. The lines and small circles show the absorption spectra of porphyropsin and cyanopsin. The photopic sensitivity agrees very well with the absorption spectrum of cyanopsin; but for reasons which are still obscure, the scotopic sensitivity is displaced about 10 mμ toward the red from porphyropsin. The lenses had been removed from these preparations; possibly

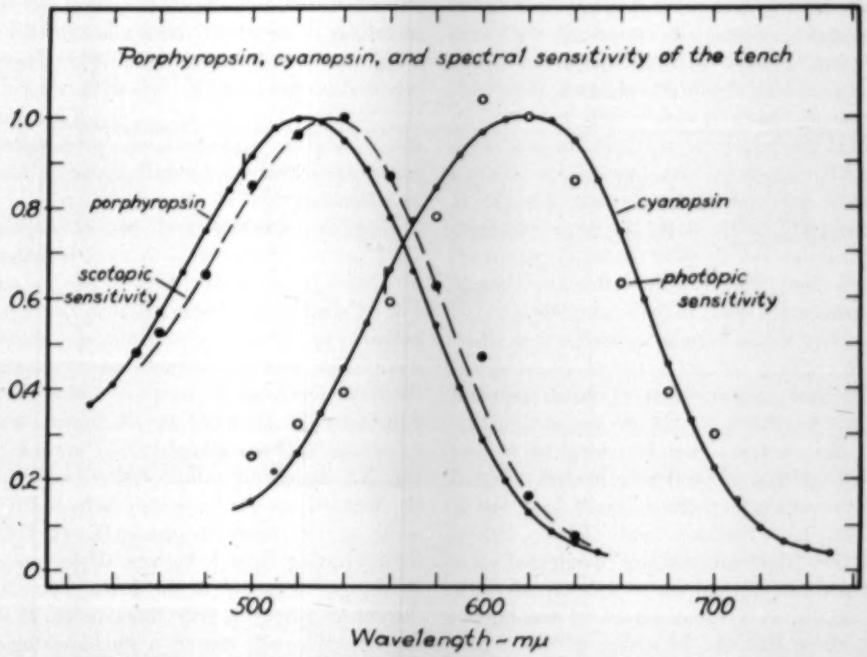


Fig. 17 (Wald). Absorption spectra of porphyropsin and cyanopsin (lines, small circles) compared with the spectral sensitivities of rod and cone vision in a freshwater fish, the tench (broken line, large circles). The spectral sensitivities were measured electrophysiologically by Granit (1941 b) in opened eyes from which cornea and lens had been removed. The photopic sensitivity agrees well with the absorption spectrum of cyanopsin, but the scotopic sensitivity is displaced about 10 mμ toward the red from porphyropsin, perhaps because of some yellow pigmentation in the retina or ocular fluids.

there was enough yellow pigmentation in the retina or in the ocular fluids to account for this discrepancy. In animals whose vision is based upon vitamin A_2 the Purkinje shift is unusually large: about 90 mμ, from about 530 mμ in the scotopic eye to about 620 mμ in the photopic eye. This shift is mimicked closely by the absorption spectra of porphyropsin and cyanopsin.

It can be concluded that in all appropriate instances, the spectral sensitivities of rod and cone vision, and hence the Purkinje phenomenon, derive directly and quantitatively from the absorption spectra of the visual pigments.

2. Visual adaptation and the bleaching and synthesis of visual pigments

For many years we have believed that some simple relation connects the visual threshold, or better its reciprocal, the visual sensitivity, with the concentration of visual pigment. In a steady illumination, the visual pigments bleach to steady state levels, maintained thereafter by regenerative processes. Simultaneously the visual sensitivity falls to a steady state value; this is light adaptation. Conversely, in the dark, the visual pigments are synthesized to their maximal concentrations. Simultaneously the sensitivity rises to a maximum; this is dark adaptation.

Lately it has become apparent that whatever relation obtains between visual sensitivity and concentration of visual pigment is not as direct as simple proportionality. On the contrary, the bleaching of a very small fraction of rhodopsin in dark adapted rods results in an extraordinarily large fall of sensitivity (Rushton and Cohen, 1954). Parallel "light adaptations" conducted on a human subject and on a solution of cattle rhodopsin in a water model of the human eye show that the bleaching of 0.006 percent of the rhodopsin lowers the visual sensitivity 8.5 times; and the bleaching of 0.6 percent of rhodopsin lowers the sensitivity 3,300 times (Wald, 1954 b). Conversely the resynthesis of the last small fraction of rhodopsin must raise the sensitivity greatly. Indeed much of light and dark adaptation in the rods seems to involve the first small fraction of rhodopsin to be bleached, and the last small fraction to be resynthesized (cf. Granit et al., 1938, 1939).

A theory has been proposed to account for this relationship (Wald, 1954 b). The rod is viewed as a compartmented structure. Each compartment contains a large quantity of rhodopsin, and is discharged by the absorption of a first quantum of light. The residual rhodopsin of a discharged compartment continues to absorb light and to bleach, but can no longer contribute to excitation. A rod is rendered wholly inexcitable when each of its compartments has absorbed at least one quantum of light, that is, when in each of its compartments at least one molecule of rhodopsin has been bleached. In this way the bleaching of very little rhodopsin can lead to a high state of light adaptation. This theory, pursued mathematically, leads to the expectation that the *logarithm* of the visual sensitivity should be approximately proportional to the concentration of visual pigment (Wald and Brown, 1954-55).

Recently Rushton and his co-workers (1955) have succeeded by a most ingenious procedure in measuring directly the rise and fall of rhodopsin concentration in the living human eye. This is a magnificent achievement. The method depends on comparing the reflection from the retina of a blue-green light strongly absorbed by rhodopsin, with an orange light scarcely absorbed by rhodopsin. No change of retinal reflectance could be detected on illuminating such rod-free areas as the fovea or the optic disc. On light-adapting areas 5 degrees, 10 degrees or 20 degrees temporal to the fovea, however, distinct increases in reflectance could be recorded, apparently caused by the bleaching of rhodopsin. The bleaching was stronger the more peripheral the field. In all cases the concentration of rhodopsin fell to a steady state level in about five minutes, in good agreement with the time known to be required for

light adaptation of the human rods (cf. Wald and Clark, 1937-38). Following light adaptation, the rhodopsin concentration rose regularly in the dark, approaching a maximum value in about 30 minutes, in good agreement with the time required for human rod dark adaptation.

The *course* of bleaching and resynthesis of rhodopsin in the human retina, measured in this way, agrees with the course of human light and dark adaptation only when the latter is plotted in terms of *log* sensitivity. It is the *logarithm* of the visual sensitivity that rises and falls with time much as does the concentration of rhodopsin. This is the relation predicted from the compartment theory of excitation alluded to above.

Another approach to the same problem extends these relationships to the cones. Figure 11 above shows a mixture of chicken iodopsin and rhodopsin, made by incubating neo-*b* retinene in solution with a mixture of cone and rod opsins. The reason the visual pigments form separately in this instance is that iodopsin is synthesized with enormously greater speed than rhodopsin—about 530 times as fast at 10°C. (Wald and Brown, 1954-55). At room temperature, the synthesis of iodopsin is complete within five minutes, while that of rhodopsin continues for well over an hour (fig. 19).

It has long been known that in man and many other animals the cones dark adapt much more rapidly than the rods. In the human eye the dark adaptation of the cones is complete within four to six minutes, while that of the rods continues for over 45 minutes. The dark adaptation of a peripheral area of the human retina containing rods and cones is shown in Figure 18. It is plotted in terms of *log sensitivity* (-log threshold), the better to expose its relationship to the rise of visual pigment concentration.

Figure 19 shows the synthesis of chicken iodopsin and rhodopsin in solution at 23°C. The data are taken from the same experi-

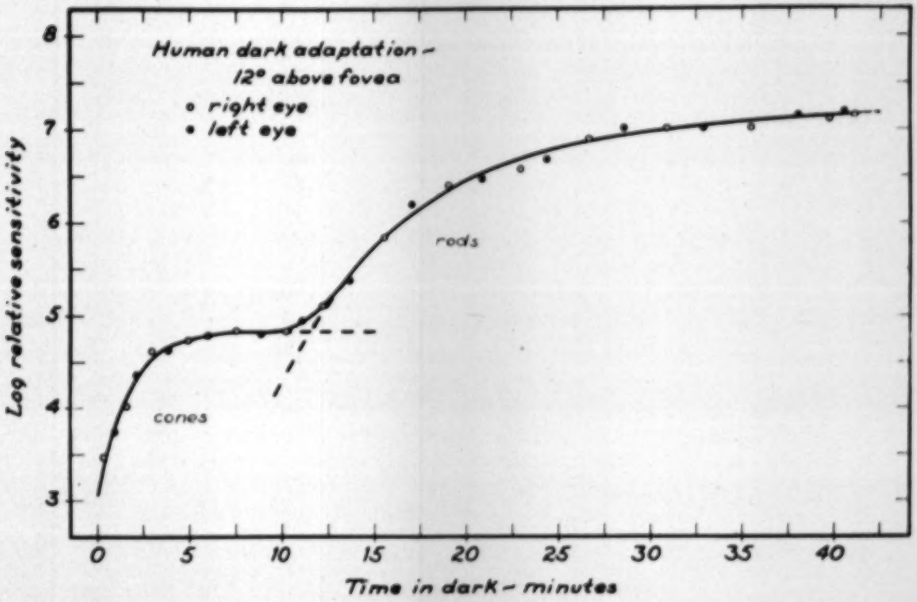


Fig. 18 (Wald). Dark adaptation of the human eye, measured in a peripheral area which contains both rods and cones. The dark adaptation of the cones is completed within about five minutes, that of the rods within about 45 minutes (from Wald, Brown, and Smith, 1954-55).

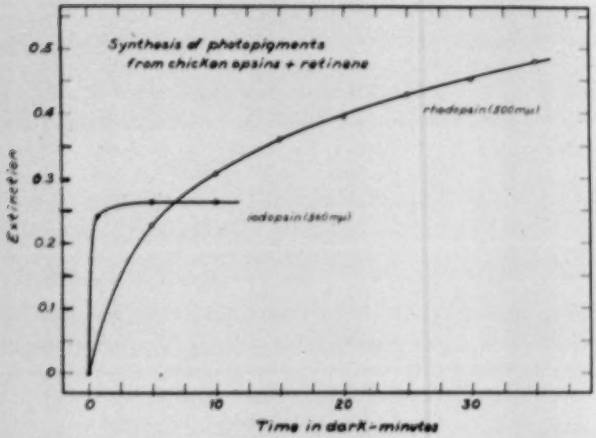


Fig. 19 (Wald). Synthesis of iodopsin and rhodopsin in solution from a mixture of chicken opsins and neo-*b* retinene. This is the same experiment shown in Figure 11, but with the rhodopsin extinctions multiplied by 1.3. At 23°C., the temperature of these measurements, iodopsin synthesis is complete within two to three minutes, whereas rhodopsin synthesis still continues after 35 minutes (from Wald, Brown, and Smith, 1954-55).

ment as Figure 12, but with rhodopsin extinctions multiplied by 1.3. It is hardly necessary to labor the close relationship between these measurements and the course of human dark adaptation, cone and rod. Again, however, what parallelism obtains involves the comparison of *log* sensitivity with the concentration of the visual pigments.

I think we can conclude from all these measurements that light and dark adaptation have their primary source in the bleaching and resynthesis of the visual pigments of the rods and cones. To be sure, more central phenomena—changes in the sensitivities of neurones and synapses along the optic pathways—may also play a role. Of this possibility we know as yet very little. In general we expect neural adaptations to be relatively rapid; if they enter at all they should probably be completed during the earliest stages of the visual adaptations we ordinarily measure. They probably are responsible also for only a minor portion of the *range* of visual adaptation. To a first approximation light and dark adaptation seem to reflect the fall and rise of visual pigment; and specifically in the form that the *log* sensitivity runs parallel with pigment concentration.

3. Vitamin-A deficiency and night blindness

Probably the earliest symptom of vitamin-A deficiency in man and other animals is the rise of visual threshold known as night blindness. Because we are used to associating night vision with the rods, it was once thought that dietary night blindness—so called to distinguish it from the idiopathic or congenital disease—is a failure specifically of rod vision. The first experimental studies of human night blindness, however, showed at once that in vitamin-A deficiency cone vision deteriorates with rod vision, and both recover together on administration of vitamin A (figs. 20 and 21) (Haig, Hecht, and Patek, 1938; Wald, Jeghers, and Arminio, 1938; Hecht and Mandelbaum, 1938).

The realization that both iodopsin and rhodopsin are synthesized from the identical forms of vitamin A appears to offer a substantial theoretic basis for this relationship. To be sure, iodopsin has not been demonstrated in human cones; if present, it is presumably accompanied by at least two other cone pigments needed to account for normal human color vision. Yet the observation that on administration of vitamin A—or carotene—night blindness is repaired as quickly and completely in the cones as in the rods (fig. 21) implies that the human cone pigments as a group are probably synthesized from the same vitamin A. Just as rhodopsin and iodopsin are constructed by joining the same prosthetic group to differ-

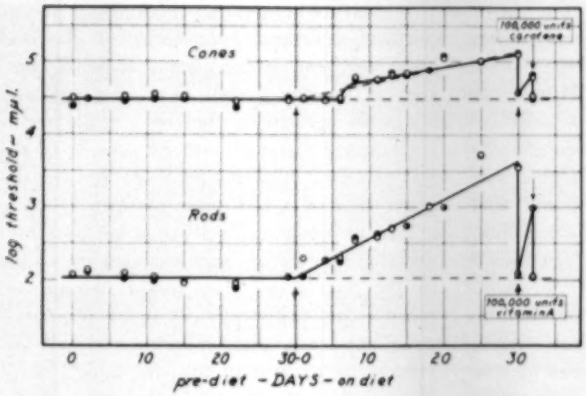


Fig. 20 (Wald). Thresholds of completely dark adapted cones and rods during 30 days of heavy vitamin A administration (left) and during 30 days on a vitamin A-deficient diet (right). Open and closed circles show thresholds of right and left eyes respectively. On the 30th day of the deficient diet, one dose of vitamin A was administered. Both cone and rod thresholds returned to normal. On the 32nd day the subject was again slightly night-blind, and was given a dose of carotene. Again both cone and rod thresholds returned to normal (from Wald, Jeghers, and Arminio, 1938).

ent opsins, so the cone pigments responsible for human color vision may well be composed of a single retinene combined with a variety of different opsins.

The opsins have been altogether a neglected component in the etiology of dietary night blindness. We have tended too much to think of this disease and its cure in terms of the removal and replacement of vitamin A, particularly since learning that this substance is a precursor of rhodopsin. Perhaps this preconception is the source of some of the embarrassment that has attended the experimental study of night blindness. The first burst of experiments which, it was hoped, might lead to a complete clarification, revealed instead two fundamental inconsistencies, neither of which has since been resolved:

1. On assuming a vitamin-A deficient diet, some subjects begin immediately to go night-blind, whereas others show no effects, visual or otherwise, for many months.

2. On administration of vitamin A to night-blind subjects, some are cured completely within several hours, whereas others retain some degree of night blindness for months of high dosage with vitamin A. Figure 20 shows the rapid type of entry into night blindness, Figure 21 the rapid type

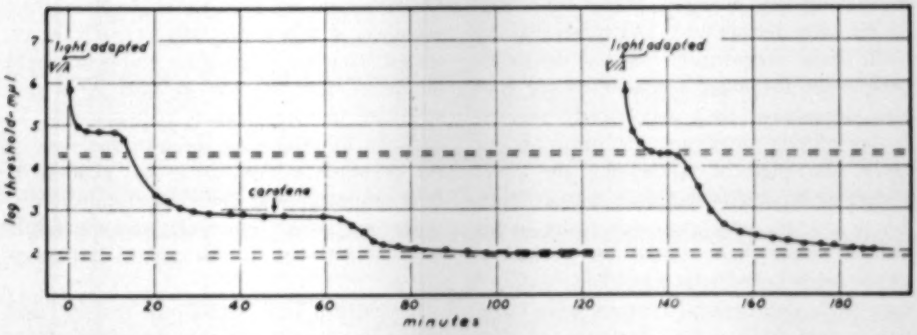


Fig. 21 (Wald). The cure of night blindness with carotene. Following a standard light adaptation, the measurement of dark adaptation shows both cone and rod plateaus to lie above their normal ranges (enclosed within the upper and lower pairs of broken lines). After dark adaptation was completed, 20,000 International Units of carotene in oil were administered in gelatine capsules, orally. For 12 to 14 minutes the rod threshold remained constant. Then it fell rapidly to normal. Immediate repetition of the standard adaptation procedure showed both cone and rod plateaus to have entered their normal ranges (from Wald and Steven, 1939).

of cure. Unfortunately the other types of result are observed at least equally often (Hecht and Mandelbaum, 1940; Wald, Brouha, and Johnson, 1942; Hume and Krebs, 1949).

I think that we have not sufficiently distinguished an acute from a chronic syndrome in vitamin-A deficiency; and that, perhaps particularly in the chronic condition, a failure of opsin rather than of vitamin A may be the predominant cause of night blindness. It must be understood that the maximal level of visual pigment synthesized in the normal retina is set, not by vitamin A, but by opsin. Vitamin A is normally always available in excess, even in mild vitamin-A deficiency. After a retina has become completely dark adapted, it contains always a small amount of free vitamin A, presumably its equilibrium level vis-à-vis the blood.

The role of vitamin A as the precursor of visual pigments seems almost trivial compared with its general role in maintaining the integrity of the tissues. The mechanism of this action is still completely obscure. In vitamin-A deficiency, various tissues all over the body begin to deteriorate, the retina among others (Tansley, 1933; Johnson, 1943). Johnson has reported that after seven to 13 weeks of vitamin A deprivation in young rats, the rods in the retinal fundus exhibit marked changes. Many outer segments have disappeared, and those that remain stain abnormally. As the deficiency progresses, the inner segments of the rods also degenerate, then successively the external limiting membrane, the outer nuclear layer, the pigment epithelium, the outer molecular layer, and the inner nuclear layer. These changes progress much faster in the central retina than toward the periphery. Outer segments of rods which have suffered only slight degenerative changes seem to repair considerably within 24 hours of vitamin-A therapy. Even rods which have degenerated completely appear to regenerate within 10 to 18 weeks of vitamin-A administration.

It should be noted that rhodopsin is an important *structural* component of the rod outer segment. It constitutes about 40 percent of the dry weight of a frog rod, and about 14 percent of that of a cattle rod—respectively about 60 and 22 percent of the rod proteins. It seems probable that the degeneration of a rod outer segment involves a loss of opsin as well as other proteins; and that long before such changes are visible in the microscope they might become detectable physiologically as night blindness.

In any case, night blindness clearly involves far more than the simple decline of vitamin A concentration in the retina. It appears to introduce, perhaps particularly in chronic deficiency, deep-seated anatomic changes, which might repair only very slowly. Associated with these we can expect fluctuations in opsin, which might on occasion be more immediately associated with the development and cure of night blindness than fluctuations in the supply of vitamin A.

In addition to deficiency in the diet, any interference with the flow of vitamin A to the retina, or with its utilization by the tissues, can be expected to react on the visual threshold. This appears to be the case in certain chronic liver diseases (cf. Patek and Haig, 1939). Bile is needed for the absorption of both carotene and vitamin A (Greaves and Schmidt, 1935). In obstructive jaundice, in which bile fails to reach the intestine, vitamin-A deficiency and hence night blindness may develop in spite of a diet adequate to meet normal requirements. In addition to producing the bile, the liver is the principal storage tissue for vitamin A. It is not surprising therefore that liver disorders may affect the extent, and apparently in some instances also the rate, of dark adaptation.

Recently it has been shown that the wall of the intestine is probably the principal site for the conversion of carotene to vitamin A (Glover, Goodwin, and Morton, 1947; Thompson, Ganguly, and Kon, 1949). It is not unlikely that conditions exist in which

some failure of this process leads to visual disturbances.

Even when the diet is adequate, and the liver and intestine performing their functions, this may not yet be enough. We know now that vision depends, not merely on vitamin A, but on a particular shape of vitamin A, the neo-*b* isomer. This is not ordinarily present in the food. Other isomers of vitamin A obtained in the diet must be bent and twisted into this special configuration. We know that this process occurs in the eye tissues; we are not yet sure that it occurs anywhere else. The neo-*b* isomer is continuously lost in the bleaching of the visual pigments, and must be continuously replaced for vision to persist. It is not impossible that there exists a visual pathology that has its source in a failure to isomerize vitamin A.

I have mentioned a further process which occurs in the eye tissues, the esterification of vitamin A. We do not yet know its role in vision. Presumably it has a role, and so may constitute another point at which the visual processes are vulnerable to disease.

The participation of vitamin A in the processes of visual excitation therefore introduces a whole series of special relationships. At this point, vision becomes dependent upon an ecological factor, the nutrition; and upon the entire network of internal arrangements that govern the absorption, metabolism, and transport of vitamin A throughout the body.

4. Nicotinamide

A second vitamin plays a basic role in the visual processes: nicotinamide, the antipellagra factor of the vitamin B complex, and the active principle of DPN, the coenzyme of the alcohol dehydrogenase system. Without this factor, so far as we know, vitamin A cannot be oxidized to retinene, the first step in the synthesis of rhodopsin and iodopsin.

Are there visual symptoms in pellagra? Is there, for example, some disturbance of dark adaptation in this disease? So far as I know, none has been reported; yet dark adaptation is not often measured in the clinic, and only large changes in this function might be noticed by the patient or become apparent to the physician. It might be well therefore to examine carefully the visual behavior of pellagrins with these considerations in mind.

Such an investigation has become difficult to pursue in the United States. In the past 20 years, pellagra has declined in this country from a persistent level of several thousand registered deaths annually to virtual extinction. To pursue this matter further at present would probably require either an ardous and problematical laboratory experiment, or an expedition. Anxious as we are to learn more of the physiology of vision in all its manifestations, I think one can only be happy concerning this particular frustration.

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STUDIES ON THE PHOTOPIC-SCOTOPIC RELATIONSHIPS IN THE HUMAN ELECTRORETINOGRAM*

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INTRODUCTION

Throughout the history of research into the electric responses of the retina the attempt has been made to correlate the explanation of these electric responses with the knowledge of the physiology of vision attained by other methods. It is indeed logical to suppose that the electroretinographic response, initiated by radiant energy, is based on the action of the two fundamental mechanisms in which vertebrate vision has its origin—the photopic and scotopic mechanisms. These two mechanisms are mediated by the cones and the rods, respectively. The outer limbs of these receptor cells, which in their physical structure in many ways resemble the nerve sheath (Wald[1]), contain photosensitive pigments, and in 1849 DuBois Reymond discovered that they are negatively charged with respect to the cornea, and for that matter with respect to all layers in front of them.

The basic chemical processes involved in the two mechanisms are the bleaching of the pigments—which somehow elicits the visual response—and their resynthesis in the dark. The quantum demand for the bleaching is intimately connected with the concentration of the pigments within the cells. The lower the concentration (or, in physiologic terms, the higher the threshold), the greater is the quantum demand (Hartline, Milne, and Wagman,[2] Rushton and Cohen[3]).

When the eye is exposed to light, a definite amount of the visual pigments is bleached. This amount is larger the higher the intensity of the light. After some time of exposure a "steady state" establishes itself, that is, an equilibrium exists now between bleached and unbleached pigments within the receptor cells.

In the dark the visual pigments are resynthesized. There is, however, a characteristic difference between the cones and the rods. The cones contain a very much smaller amount of photosensitive pigments than the rods and their resynthesis is completed after three or four minutes, while the complete resynthesis of the rod pigment, the rhodopsin, requires almost one hour (Wald, Brown, and Smith[4]).

The dark-adaptation curve clearly shows the differentiation between the cone and the rod mechanism. By using an adequate preadaptation, that is, by proper bleaching, it is possible to knock out the scotopic mechanism for a period of time, the length of which depends on the intensity and the duration of the preadapting light. Since the visual threshold is governed by the response of the most sensitive elements one is thus able to obtain during the first few minutes of dark adaptation a pure photopic response (the cone curve). As the dark adaptation proceeds further, the scotopic response dominates the dark adaptation curve more and more (Auerbach and Wald[5]).

While there is a striking parallelism between the chemical reactions, both in vitro and in vivo, and the visual thresholds during dark adaptation, it must be stressed that the

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photopic and scotopic mechanisms as a whole cannot be expressed solely in terms of chemical reactions. The chemical processes in the receptor cells which follow their stimulation by a light are trigger mechanisms which set off a neural process. It is in this broader sense that we shall use the terms photopic and scotopic mechanisms in this paper.

The known and accepted form of the human electroretinogram is said to be made up of two components: a negative component with a very short latent period (the a-wave), and a positive component with a longer latent period (the b-wave). In other vertebrates than man there is a third and even later positive deflection (the c-wave), and an off-effect can also be noted. Thus the electroretinogram consists of a succession of different waves and various attempts have been made to explain and analyze them, beginning with Einthoven and Jolly,[6] and Piper[7] who interpreted the electroretinogram by assuming that it is made up of the algebraic summation of a number of hypothetical individual components or processes.

In 1917 Tirala[8] published experiments in which he had attempted an analysis of the electroretinogram by means of various anesthetics and other drugs, but his methods were still not refined enough to enable him to come to clear-cut conclusions. Granit,[9] using also pharmacologic methods, established that there appear to be three distinct processes (P I, P II, P III) the algebraic summation of which is the electroretinogram. Granit showed that with increasing depth of anesthesia the three processes disappear successively from the electroretinogram.

In 1942 Motokawa and Mita,[10] working with orange-red test stimuli, demonstrated in man two different positive waves the first of which they called the X-, the second the b-wave. Their nature was explained by Adrian[11] who discovered the phenomenon independently in 1945, and who also obtained two positive waves which were most distinct when he used orange-red lights. Adrian conceived them as belonging to the photopic and the scotopic mechanism, respectively, and ascribed the first positive wave to the photopic and the second positive wave to the scotopic response. Armington[12] and Schubert and Bornschein[15] came to a similar conclusion. The latter two investigators proved definitely that the X-wave is due to cone response by showing that it is obtained by the use of red test light above 600 mμ in normal, hemeralopic, and deuteranopic subjects, but not in protanopes.

In 1952 Armington, Johnson, and Riggs[13] showed that the a-wave also contained two components, one photopic and the other scotopic. The best responses were again obtained with red lights. Best,[14] working with white test stimuli, concluded that both the a- and the b-wave are to be considered as the response of both types of receptor cells.

In the same year Schubert and Bornschein[15] mentioned for the first time a hump in the descending branch of the b-wave of the human electroretinogram. A year later Bornschein[16] published a figure in which he superimposed the responses of different dark adaptation periods. In this figure there appeared a distinct hump climbing up on the descending branch of the b-wave during dark adaptation. However, these authors did not discuss this hump in either paper. In 1954 Burian[17] stressed the importance of this hump and the implications of its upward climb during dark adaptation. He emphasized that the positive b-wave is a composite response of both the photopic and the scotopic mechanisms.

As for the specific retinal elements to which the components of the electroretinogram may be assigned, various assumptions have been made. Granit[9] believed that the process causing the b-potential was localized in the synaptic layers of the retina and Bartley[18] suggested that the a-wave might be due to a response of the receptor cells, the b-wave to a response of the bipolars. From clinical evidence it is unlikely that the gan-

glion cells are necessary for the appearance of an electroretinogram since it can be obtained from eyes which have been blind from glaucoma for some time. On the other hand, the presence of intact receptor cells is essential. This is obvious both from the clinical evidence with retinitis pigmentosa and from the evidence obtained in degenerated rod-free retinas of mice (Keeler, Sutcliffe and Chaffee[19] and Keeler[20]) and in the experimental studies of Noell[21] with agents causing selective damage to retinal receptor cells.

The studies reported in this paper were made with a view to investigating further the photopic-scotopic relationships in the human electroretinogram and to assigning more securely, if possible, its various components to specific retinal structures.

Apparatus and Procedure

Our experimental set-up is schematically shown in Figure 1. The subject was placed in an electrically shielded cage, facing the flash lamp of a Grass Photo-stimulator (Model PS-1) through a small window, his chin on a chin rest. The electrodes used were of the type described by Burian.[22] The corneal electrode consisted either of a silver or stainless steel wire ring of a diameter of approximately 10 mm.

The indifferent electrode was fastened to the supraorbital area and the ground electrode to the ear. The electrodes were connected to an ink-writing Grass electroencephalograph (Model III D) with a paper speed of 120 mm. per second in all experiments. This speed was chosen to stretch the response, thus obtaining its details more clearly. Using the AC amplification of the Grass instrument we led the electrodes through a condenser to a DuMont Cathode-Ray Oscillograph (Type 322-A).

The second channel of the electroencephalograph was connected with a photoelectric cell which was fastened opposite the flash lamp of the stimulator.

Facing the screen of the oscillograph was a Grass Kymograph Camera (Model C-43); the stimulator activated both the oscillograph and the camera. The complete system was thus synchronized.

A manual switch initiated the following events: the flash appeared and the resulting retinal action potential was recorded both by the pen of the electroencephalograph and by the oscillograph; simultaneously the shutter of the camera was released to photograph the response on the oscilloscope.

We used two recording systems because we could not obtain all details of the retinal response by the pen-paper arrangements of the electroencephalograph. The vertical movements of the pens were restricted, so that they could not register a complete re-

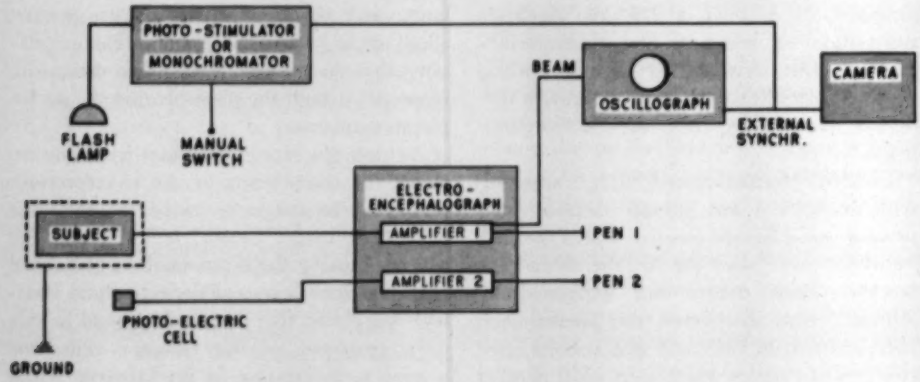

Fig. 1 (Auerbach and Burian). Block diagram of experimental set-up.

sponse if the amplitude had grown too high during dark adaptation. By decreasing the gain, one obtained, of course, the complete response, but at the expense of clarity of detail. An additional disadvantage was the inertia of a paper-pen arrangement. There were no such disadvantages when the response was recorded electronically by an oscillograph. However, the ink recording system gave us the opportunity to recognize immediately the approximate shape of the response and irregularities caused by eye movements. In addition it provided a convenient method of taking notes.

Before each experimental session the gain of the amplifier was calibrated and photographed. The 60 cycles of our power source were also photographed to obtain a measure of the time relations.

The Grass Photo-stimulator provides five different intensities of the stimulating light which are indicated on the machine as relative intensities, each step doubling the intensity of the previous one. The lowest luminance (indicated on the stimulator as 1) we measured to be 1.29 millilambert. The highest luminances which we could use with this machine was 20.64 mL (indicated on the stimulator as 16). In most cases we employed an intensity of the stimulating light of 2.58 mL and of 5.16 mL. The reflector of the flash lamp of the stimulator is parabolic in shape with a diameter of 13 cm. The exposure time was approximately 10 to 15 μsec. The instrument was placed at a distance of 30 cm. in front of the subject and a fixation light of adjustable intensity was provided. The response of the Grass capacity coupled amplifier decayed 50 percent in approximately 1/10 sec.

At the beginning of each experiment the subject was preadapted in one of two ways. In the first procedure we used the reflected light of the sphere of the Goldmann adaptometer with a luminance of approximately 2,650 mL. The second procedure was the same as that described by Auerbach and Wald[5] with the exception that a 500-watt instead of a 1,000-watt lamp was used at a distance of 65 cm. from a plano-convex lens (focal length 10 cm., diameter 9.0 cm.). The eye was held in the position of Maxwellian view. This last procedure was used for both white and colored light adaptations. The brightness of the adapting lights was measured with a Macbeth Illuminometer. After 10 minutes the light was turned off and the eye was stimulated as soon as possible.

We began another experimental series using colored test lights combined with white or colored preadaptations. The results of these experiments will be reported elsewhere. But since one result will be included in this paper, a short description of the experimental arrangement is given. We employed two methods to produce monochromatic light. The first consisted of a prism monochromator, using as light source a 300-watt Sylvania zirconium concentrated arc lamp. The brightness was adjusted by a movable neutral wedge. The exposure times were regulated by a photographic shutter released through a solenoid. The monochromator was included as an independent light source instead of the Grass photo-stimulator (fig. 1). Again a manual switch initiated the whole system.

Alternatively, Wratten color filters were placed in front of the eye by means of a goggle. Here the Photo-stimulator was employed.

In another series of experiments, electroretinographic readings were taken at different intervals during adaptation to 25-, 60-, 100-, 200-, and 500-watt lamps. Measurements of the luminances were again made with the Macbeth Illuminometer (table 1).

TABLE 1

LUMINANCES OF BULB BEHIND OPAL GLASS IN ONE-METER DISTANCE

	Cdl/ft^2	mL
25 Watt	386	1,306
60 Watt	925	3,128
100 Watt	1,545	5,225
200 Watt	3,090	10,450
500 Watt	7,725	26,125

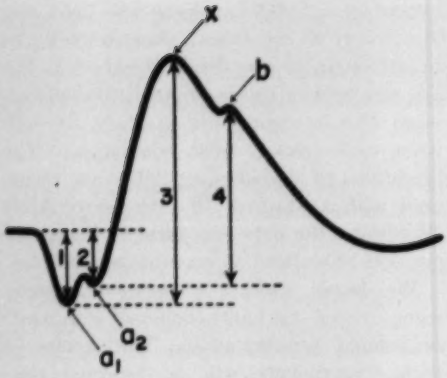

Fig. 2 (Auerbach and Burian). Schematic presentation of an electroretinogram: 1 and 2, way of measuring the negative waves a_1 and a_2; 3 and 4, way of measuring the positive waves X and b.

The following arrangements were made: the subject was dark adapted for a period of 10 minutes. Then the adapting light, beginning with the 25-watt lamp, was turned on behind an opal glass at a distance of 1.0 m. from the subject. The stimulating flash light was placed between the subject and the opal glass at a distance of 30 cm. from the subject, and was flashed into the observer's eye at intervals of 30 to 60 seconds with the adapting light left on. After completion of the experiment with surrounding illumination obtained by the 25-watt lamp, the next stronger lamp was turned on and the same experiment repeated on this new level of light adaptation.

The measurements of the recorded responses on the film were made with the help of a microfilm reader. The negative waves of the response were measured from the base line. The first positive wave was measured from the peak of the first negative wave, the second positive wave from the peak of the second negative wave (fig. 2).

Results

1. *The electroretinographic changes during the course of dark adaptation.* Burian has shown in 1954[17] that a small hump on the descending branch of what is customarily called the b-wave has the tendency to climb upward during the course of dark adaptation. On closer examination we found that the beginning and the rate of this climb depend on both the intensity and the duration of the light adaptation. The lower its intensity and the shorter its duration, the higher is the initial amplitude of this hump at the beginning of dark adaptation and the faster is the rate of climb (fig. 3).

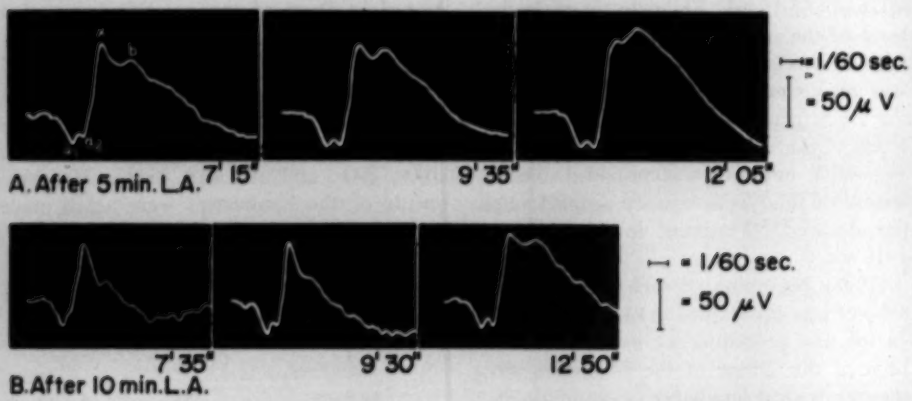

Fig. 3 (Auerbach and Burian). C. W., November 11, 1954. Light adaptation: 2,650 mL.; test light: 2.58 mL. Comparison of electroretinograms; A: after five minutes of light adaptation; B: after 10 minutes of light adaptation. The shorter the duration of light adaptation the higher is the initial amplitude of the b-wave at the beginning of dark adaptation.

a. *The two positive elevations*

In our experiments, using white test lights ranging from 1.29 to 5.16 mL, we always obtained in the electroretinogram two positive elevations with slightly different latent periods: 60 to 68 msec. for the first, 80 to 85 msec. for the second elevation (measured as peak latencies).*

From the beginning of the dark-adaptation period the first wave appears as a peak of already significant amplitude which grows during the next three to five minutes; the second elevation appears either as a slight hump on the descending branch of the first wave or isolated from the first wave as a separate peak. More often it is of the first type. This second positive wave is at first very near the base line or even below it, depending on the degree of light adaptation preceding dark adaptation.

During the following 10 minutes of dark adaptation this second wave climbs steadily upward until it reaches the same amplitude as the first wave (fig. 4). From this stage on there are two possibilities of further development: (fig. 5) the second wave either remains separate from the first one, surpassing it in amplitude, or (fig. 6) it swallows the first wave so that apparently only a single wave continues the further growth; but even in the latter case one can often detect the presence of the first wave as a slight notch on the ascending branch of the resultant composite wave. These positive elevations are followed by a slow negative wave, sometimes overshooting the baseline which grows smaller in the course of dark adaptation (fig. 4).

It must be stressed that the amplitude of the first positive elevation increases for approximately three to five minutes, after which it levels off, increasing only slightly during the next five to seven minutes. The amplitude of the second elevation is initially very low but increases steadily after about two minutes in the dark. These relations are shown graphically in Figure 7. After approximately 10 minutes the curves representing the amplitudes of the two waves cross, and the curve of the second wave goes rapidly downward, indicating a rapid increase in ampli-

* After the onset of the stimulus, there is a short period (approximately 20 msec.) during which no response is apparent. This is the true "latent period." We have, however, not singled this out but instead measure the time from onset of stimulus to peaks of response. We call these the "peak latencies."

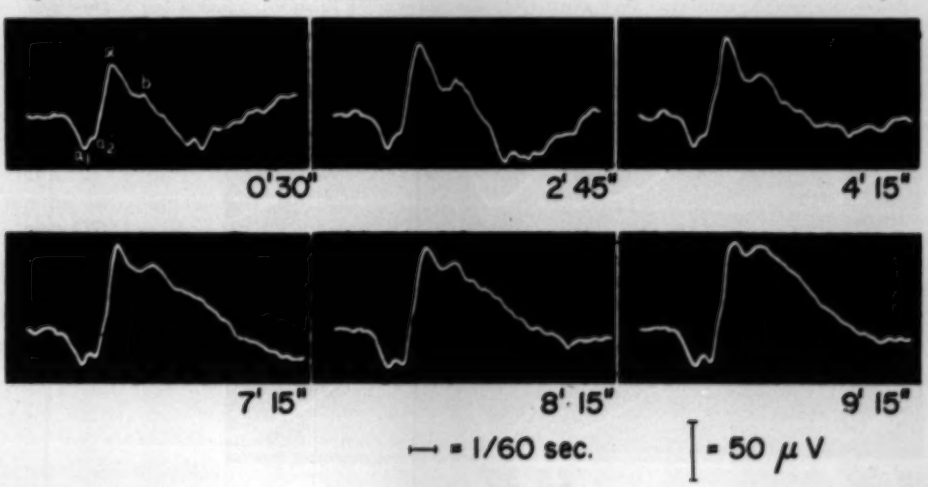

Fig. 4 (Auerbach and Burian). C. W., November 11, 1954. Light adaptation: 2,650 mL; test light: 2.58 mL. Electroretinographic response during dark adaptation up to the time at which a_r is approximately equal to a_s, and X to b.

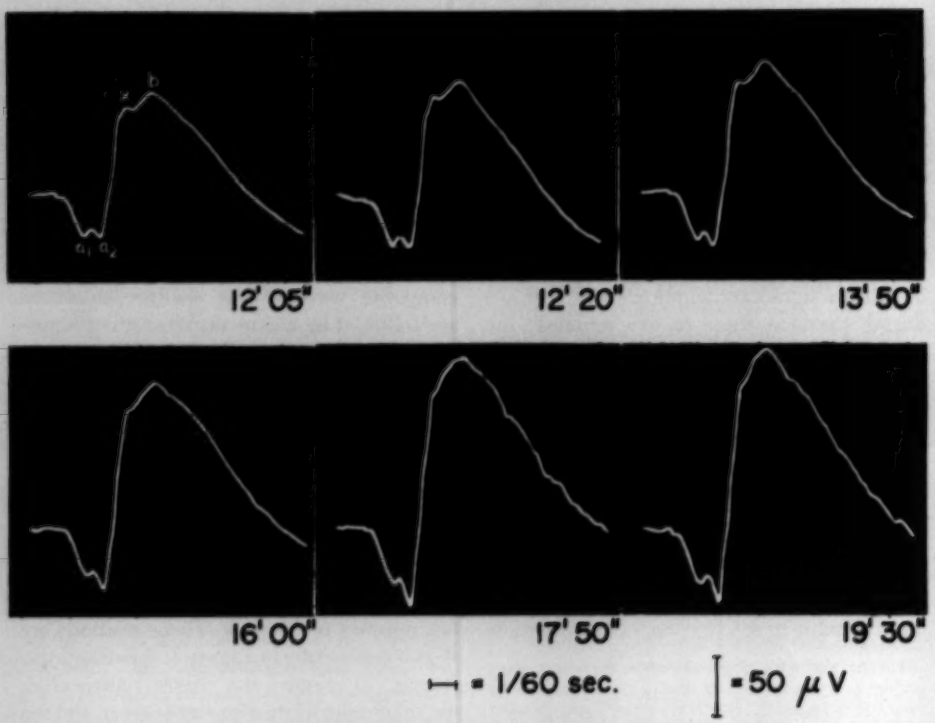

Fig. 5 (Auerbach and Burian). Continuation of Figure 4. Electroretinographic response during further course of dark adaptation. $a_1 < a_2$, X<b. The more dark adaptation progresses, the more grow a_2 and b relative to a_1 and X, until after approximately 10 minutes a_2 and b begin to surpass a_1 and X (first type: X and b remain separated during whole dark adaptation).

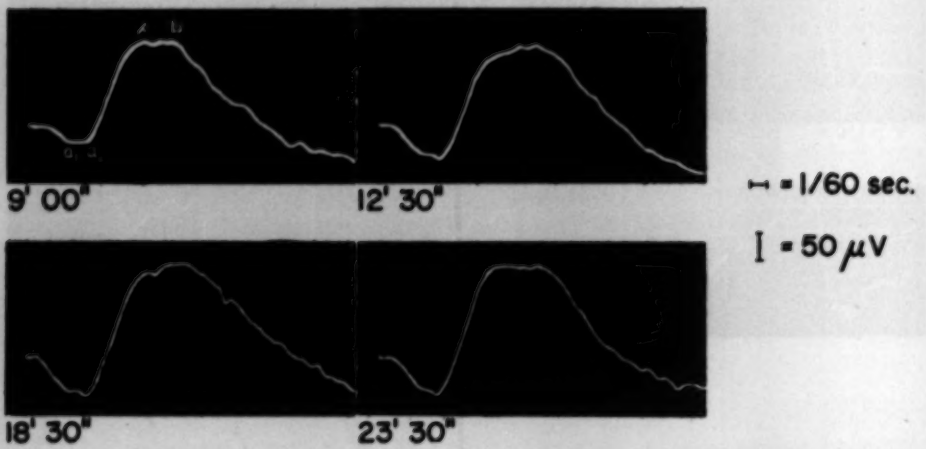

Fig. 6 (Auerbach and Burian). Ch. G., May 4, 1955. Light adaptation: 2,650 mL; test light: 2.58 mL. Electroretinographic response during a later period of dark adaptation at which $a_1 < a_2$ and X<b. The X- and b-wave have merged together in contradistinction to Figure 5 where X and b remained separated (second type).

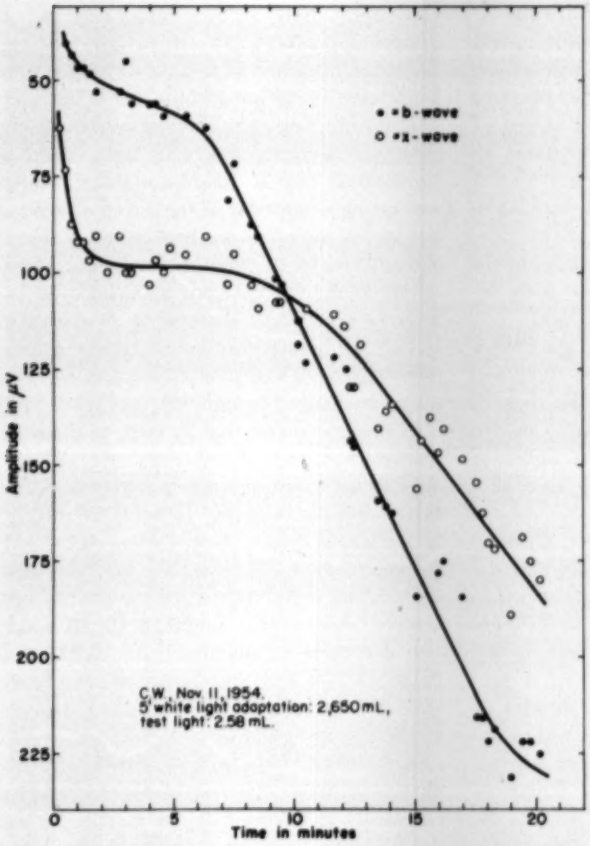

Fig. 7 (Auerbach and Burian). Graphic representation of the relation of the amplitudes of the X- and the b-waves during dark adaptation. In the majority of records the b-wave does not grow during the first two to three minutes in the dark.

tude. But also the curve representing the first wave changes its slope, and goes downward, but not as rapidly as the second curve, showing that here, too, there is an increase in amplitude.

b. *The two negative waves*

In all our experiments there appears from the very beginning of dark adaptation a negative wave of relatively high amplitude with a short peak latency (30 to 38 msec.), much shorter than that of the first positive elevation; this is the a-wave. But there also appears shortly after the adapting light is turned off a second negative wave of very low amplitude and slightly longer latency (44 to 49 msec.). The growth of the amplitude of the first negative wave reaches a temporary standstill after some minutes, but the second wave begins to increase in negativity, finally surpassing the first negative wave (figs. 4 and 5).

c. *Relationship between the two positive and the two negative waves*

For purposes of identification we shall designate the two negative waves as a_1 and a_2, and shall call the first positive elevation the X-wave and the second positive elevation the b-wave (fig. 2). In our discussion we shall try to justify this designation.

There is a striking parallelism in the growth of the two second waves, the a_2 and the b-wave: the a_2 increases its negativity

roughly at the same rate with which the X-wave increases in positivity. Approximately at the time when the b-wave has reached the height of the X-wave, a_2 has reached the same amplitude as a_1. During the later stages of dark adaptation a_2 invariably surpasses a_1, and b surpasses X (figs. 4 and 5).

2. *The electroretinogram at various intensities of test light.* As Figure 8 shows there is a marked increase in the amplitudes of the negative and positive deflections with increase in the intensity of the test light.

With the strongest test lights of the Photostimulator having a luminance of 10.32 and 20.64 mL, a_2 and b show for the first 10 minutes of dark adaptation the same growth of their amplitudes as with test lights of lower intensity; however, a_2 and b never surpass a_1 and X.

3. *The electroretinogram at different levels of light adaptation.* We shall now describe the results obtained in experiments in which the subjects were exposed to different levels of illumination (table 1 and fig 9). When the surrounding illumination is provided by a 25-watt bulb the amplitudes of both positive and both negative waves decrease for a period of five to eight minutes (the duration depending on the length of time the subject was in darkness prior to the experiment). After this an electroretinogram establishes itself with a rather low a_1, a shorter a_2, a large X-, and a low b-wave. This picture remains constant as long as one continues the experiment.

If one now increases the surrounding illumination by using a 60-watt bulb, again a transitional period follows with gradually decreasing amplitudes of the different waves, but lasting only two to three minutes. Then another electroretinogram establishes itself which differs from the one obtained with the lower intensity of surrounding illumination in that the b-wave has a much lower amplitude. Again this new electroretinogram remains unchanged indefinitely.

If one continues, step by step, to increase the surrounding illumination, one obtains always short transitional periods in which the amplitudes decrease, followed eventually by a response which always remains constant for each level of illumination. Illuminations up to about 3,000 mL depress primarily the b-wave. Above this intensity the X-wave also decreases in amplitude. At 10,000 and 26,000 mL the b-wave is absent or very small and almost exactly at the level of the trough of the a_1.

4. *Effect of intermittent flashes during dark adaptation.* If one stimulates an eye repeatedly at intervals of one second during the first five to seven minutes in the dark,

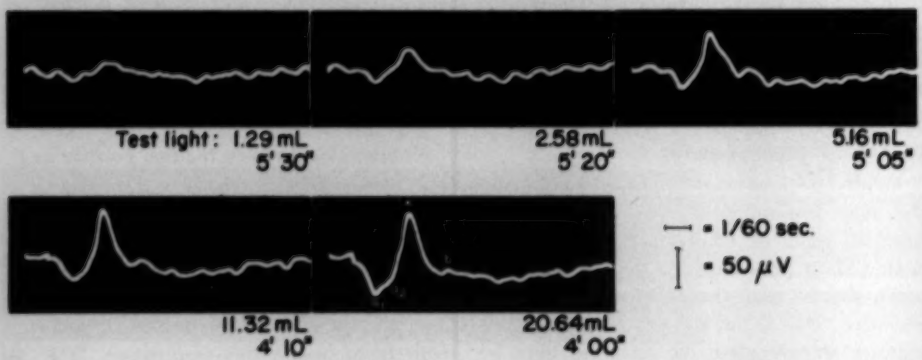

Fig. 8 (Auerbach and Burian). L. B., December 10, 1954. Light adaptation: 2,650 mL. Electroretinographic response to different intensities of test lights at approximately the same period of dark adaptation, showing an almost linear increase of amplitudes with increasing intensity of test light.

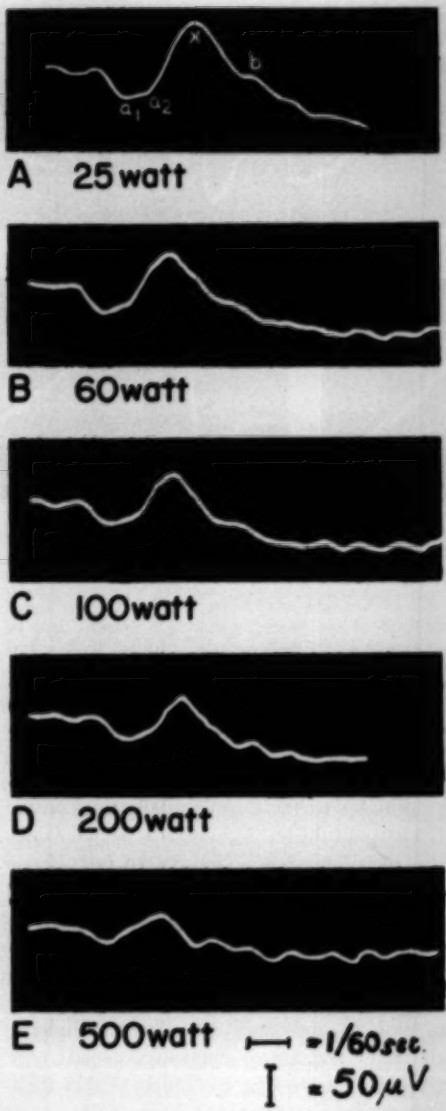

Fig. 9 (Auerbach and Burian). L. A., April 10, 1955. Test light: 2.58 mL. Electroretinographic response at various levels of light adaptation. For each level of surrounding illumination (A-E) a steady state is established.

the period in which the increase of the amplitudes of both second waves (the a_2 and the b) have not yet reached the height of the two first waves (the a_1 and the X), almost no change occurs in the electroretinogram by stimulation at all five levels of test light intensity.

But if one flashes the stimuli at later stages of dark adaptation, beginning from the time in the dark when the two positive as well as the two negative waves are approximately of equal height, the b-wave drops markedly, whereas the X-wave maintains its amplitude. When the flashing stimuli are stopped, it takes at least 25 to 30 seconds for the response to return to where it was before the flashing started (fig 10).

The higher the intensities of the stimulating light during the later stages of dark adaptation the more accentuated is this phenomenon, and with 20.64 mL the b-wave disappears entirely and the X-wave is diminished to a very small amplitude. Also here the previous status quo is reestablished after an interruption of at least 30 seconds.

5. *The electroretinogram during dark adaptation, obtained by the use of red test lights.* The results of these experiments are described at the end of the section "Discussion." Their inclusion in this paper proved to be necessary out of controversial reasons for clarification of both our results and an apparent disagreement.

Discussion

We have presented above the change in amplitude which the four components of the electroretinogram (a_1, a_2, X, and b) undergo during dark adaptation (fig. 11). It is apparent from our findings that there is a close parallelism in the growth of the amplitudes of a_2 and b in relation to a_1 and X during dark adaptation and one is, therefore, justified in assuming that a_2 and b are the electrical expression of one process, and a_1 and X the expression of another. Because of their behavior during the course of dark adaptation we believe that a_2 and b represent in effect the response of the scotopic mechanism, whereas a_1 and X represent the photopic mechanism. Thus we are dealing with two

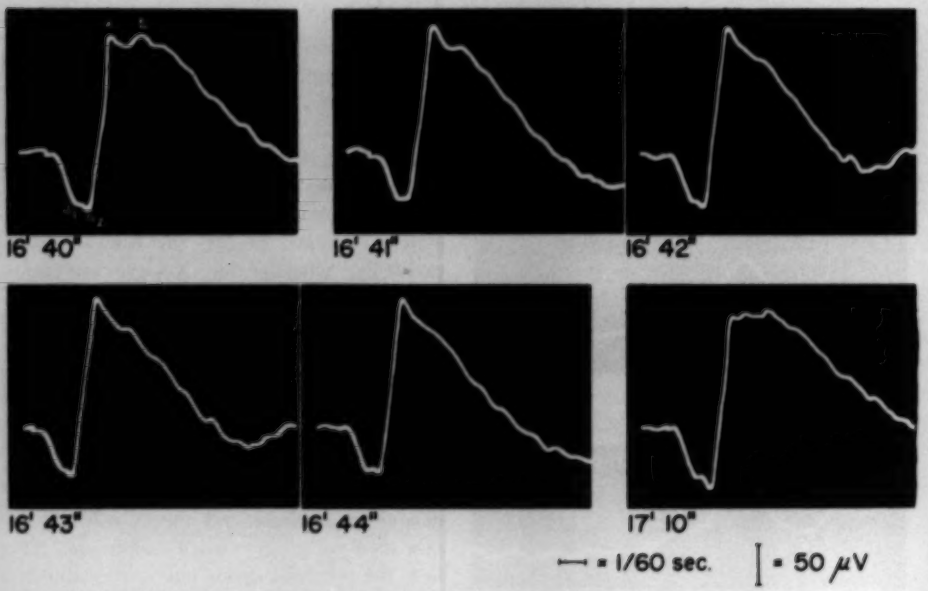

Fig. 10 (Auerbach and Burian). L. B., December 15, 1954. Light adaptation: 2,650 mL; test light: 5.16 mL. Series of intermittent flashes separated by interval of one second. Showing a marked drop of the b-wave; after an interruption in the dark the status quo is re-established.

curves, partly overlapping because of different latencies, in which the negative waves a_1 and a_2 are correlated with their positive counterparts X and b.

A further analysis of our findings shows that it is possible to ascribe at least tentatively the various components of the electroretinogram to the different parts of the photopic and scotopic mechanisms.

The peak latencies of a_1 and a_2 are considerably shorter than those of X and b. The negative waves, therefore, arise at an earlier stage in the conduction process, and conceivably in the photoreceptor elements themselves. Since a_1 is large from the onset of dark adaption and grows in amplitude during the first three to five minutes, while a_2 is very small or absent at the very beginning and grows considerably during the later phases of dark adaptation, we attribute a_1 to the cones and a_2 to the rods. It is reasonable to correlate the behavior of the two negative waves during dark adaptation with the content of resynthesized pigment in the receptor cells and with their ability to absorb quanta of light. The experiment in Section 2 aimed specifically at showing the correlation of the amplitudes of the negative deflections to the intensity of the test light clearly demonstrates this interdependence (fig. 8). In fact, if one were to succeed in improving the electrical recording method, one might be able to measure the relative numbers of quanta absorbed by the two kinds of receptor cells.

Since X and b have longer latencies than a_1 and a_2, and since they have a diphasic character and are of opposite potential with respect to a_1 and a_2, we conclude that these two waves are triggered by a_1 and a_2 and may represent the response of the intraretinal nervous pathways. The rate of impulse conduction measured by peak latencies for a_1:X and for a_2:b is almost exactly equal (approximately 1:2). What accounts for the different latencies of the two mechanisms we cannot at present explain. One possible ex-

planation may be sought in the one-to-one connection of the central photopic system as compared to the converging peripheral system with its many opportunities for delays.

The views proposed here are further strengthened by the observations in Section 4 (series of flashes separated by an interval of one second) (fig. 10). Here we tried to show the mutual relationship between the amount of visual pigment within the receptor cells and the intensity of the test light, that is, the light adapting influence of the test light. The apparent stability of the response during the earlier stages of dark adaptation, a period in which the a_2 and the b-wave have very small amplitudes, we explain by the fact that the amount of rhodopsin is so small that the changes in concentration produced by the light adapting effect of these repeated flashes cannot be registered in the electric response. The fact that we never obtained at this stage a significant drop of the b-wave with the intensities of the test light used simply implies that we were in a steady state. However, in the later stages of dark adaptation in which much more rhodopsin has resynthesized, the scotopic b-wave was markedly affected and dropped till a steady state was reached. With the higher intensities of the test light (10.32 and 20.64 mL) both the photopic X- and the scotopic b-wave dropped very much. In fact with the latter intensity the b-wave disappeared completely, and the X-wave dropped to a very small amplitude until a new steady state was established.

Further evidence for the scotopic nature of the b-wave is to be found in the fact that the height of its initial response depends on the intensity and duration of the light adaptation preceding the dark adaptation. The less thorough the pre-adaptation, that is, the less intense and the shorter it is, the higher will be the initial response of the b-wave (fig. 3).

Because of the dependence of the initial threshold of the scotopic mechanism on the intensity of the preceding light adaptation, one must assume that dependent on, and related to, the surrounding illumination an equilibrium is established between rates of bleached and unbleached or resynthesizing visual pigments, since a "steady state" is maintained so long as it is not disturbed by a change in the surrounding illumination.

This is clearly demonstrated in the electroretinographic response obtained with different levels of surrounding illumination (fig. 9). Following each increase in surrounding illumination a steady state establishes itself which remains indefinitely, as long as the level of illumination is maintained, and which is characteristic for the particular level of illumination. The stronger this illumination, the smaller the amplitude of the b-wave. At a level of approximately 3,000 mL of surrounding illumination the amplitude of the b-wave drops to a minimum. If higher intensities are employed, there is now also a reduction in the amplitude of the X-wave, an indication of the light adapting influence of the surrounding light on the photopic mechanism.

In the light adapted state the photopic mechanism is more sensitive than the scotopic. The sensitivity of this latter mechanism increases slowly during the process of dark adaptation to become finally many times more sensitive than the former. In the dark adaptation curve obtained by the psychophysical method one uses the trick of knocking out the rod response for some time by means of an adequate light adaptation. Since the threshold of the rods is higher and their rate of adaptation slower than that of the cone pigments, one is able to separate the responses of the two mechanisms and to obtain a photopic and a scotopic phase in the dark adaptation curve.

By the very nature of the psychophysical method of determining the dark adaptation curve one is unable to obtain the scotopic response during the photopic phase. In other words, it is impossible by this method to obtain a rod response as long as its threshold remains higher than that of the cones.

However, with the electroretinographic method one is able to stimulate both types of receptor cells simultaneously provided the intensity of the test light is strong enough. The electrical method provides us with both responses separately and simultaneously to the degree to which the different photopigments have been resynthesized and this also during the photopic phase of dark adaptation. One is thus able to obtain the initial thresholds of both the photopic and the scotopic responses (fig. 7).

All this is seen clearly in the behavior of the different components of the electroretinogram (figs. 4, 5, and 11). As long as the scotopic threshold is higher than the photopic, one obtains a photopic response of greater electromotive force than that of the scotopic response, but both responses are present almost from the beginning. During the first phase of dark adaptation a_1 and with it the X-wave are of higher amplitude than a_2 and the b-wave. This means that the response of the scotopic mechanism only slightly interferes with that of the photopic mechanism by overlapping. In the course of dark adaptation the scotopic response grows; a_1 and a_2, and X and b first become equal in amplitude and finally a_2 and b exceed a_1 and X, respectively. The growing scotopic response curve more and more overlaps the photopic X-wave, superimposing its electromotive force, and the true shape of the two waves is falsified and distorted by the superposition.

The consequence of this summation is that the true electromotive force of each of the two processes at this stage must differ from

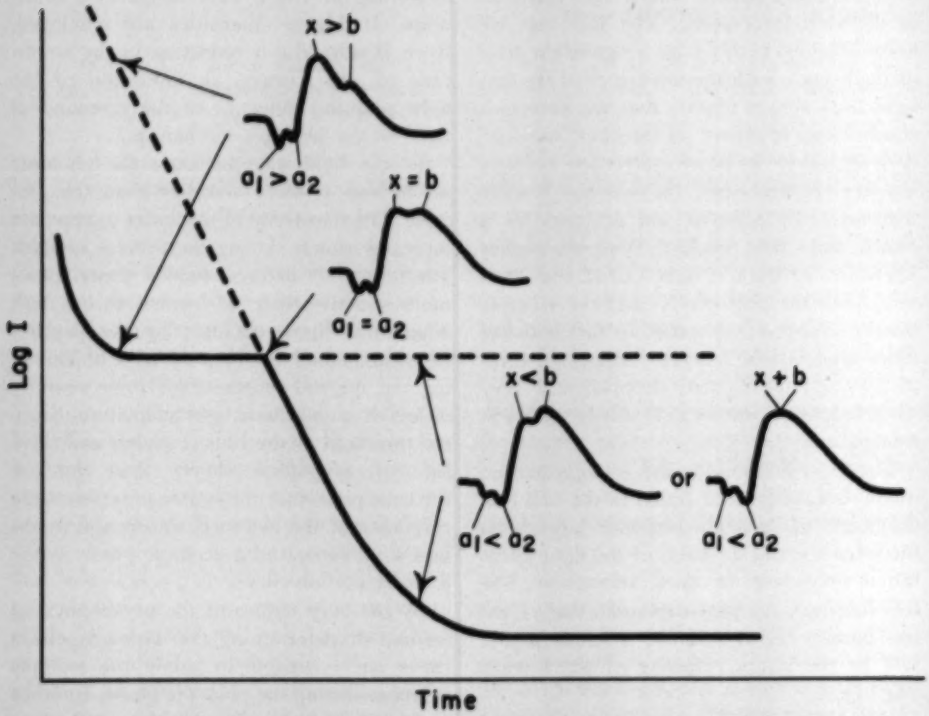

Fig. 11 (Auerbach and Burian). Schematic presentation of dark adaptation curve with corresponding diagrammatically represented electroretinograms showing relationships of a_1, a_2, X, and b during the different phases of dark adaptation.

the one recorded in the electroretinogram, and that the true maxima in each reading are attained at different times than would appear from the raw data. A first schematic attempt at a possible re-interpretation of the raw data by analysis into two partial curves representing the individual photopic and scotopic mechanism is given in Figure 12.

It is also evident from this presentation that the electroretinographic dark adaptation curve cannot be entirely correct, if it is obtained by measuring only the first positive elevation which later becomes the combined X- and b-wave, disregarding the growing positive second elevation, as has been done by some authors (Karpe and Tansley,[23] Riggs et al.,[24] Best,[14] Alpern and Faris[25]). Measurements taken in this way yield a dark adaptation curve the first plateau of which is actually represented mostly by the cone response, at least during the first two or three minutes in the dark where the scotopic component is adding only an inappreciable amount of electromotive force. But the steep slope following this plateau represents the summation of the electromotive forces of the photopic and scotopic mechanisms.

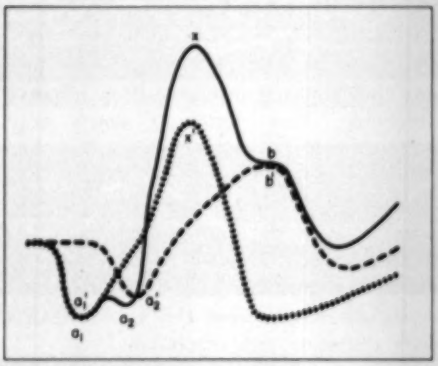

Fig. 12 (Auerbach and Burian). A possible analysis of raw data:
a_1 and a_1^1: cone response
a_2 and a_2^1: rod response
X and X^1: response of intraretinal photopic pathway
b and b^1: response of intraretinal scotopic pathway
a_1 a_2 X b: electroretinographic reading
a_1^1 X^1: individual photopic mechanism
a_2^1 b^1: individual scotopic mechanism

By the isolated measurement of the responses of the two visual mechanisms from the raw data a truer picture is obtained: the photopic response (either a_1 or X) forming a plateau after approximately three to five minutes and the scotopic response (either a_2 or b) beginning with its initial threshold and crossing the photopic plateau after about 10 to 15 minutes (fig. 7). But the electroretinographic dark adaptation curve obtained by measurements which consider the different components, X, b, a_1, and a_2, from the raw data can also not give the correct dark adaptation curve because of the summation of the individual electromotive forces resulting from overlapping. The construction of a true electroretinographic dark adaptation curve will, therefore, have to await the mathematical analysis of the experimental data.

Some comments must be added here concerning the evaluation of results obtained by the use of orange-red test lights. As has been mentioned in the introduction to this paper, Adrian,[11] and later also Armington[12] and Schubert and Bornschein,[15] found responses with two positive waves using a red test light.

We performed a series of experiments using monochromatic light obtained both from a monochromator and from color filters. The details of our results will be published later elsewhere. We wish here only to report experiments with Wratten color filters *22*, *29*, and *70*.

1. With Wratten filter *29* (having a spread from about 610 mμ to the long wavelength end of the visible spectrum and a dominant wavelength at 632 mμ) we obtained electroretinographic responses with three positive waves. The behavior of the first and second positive wave was identical with that of our X- and b-wave respectively after stimulation with a white test light; they had in addition the same peak latencies. We

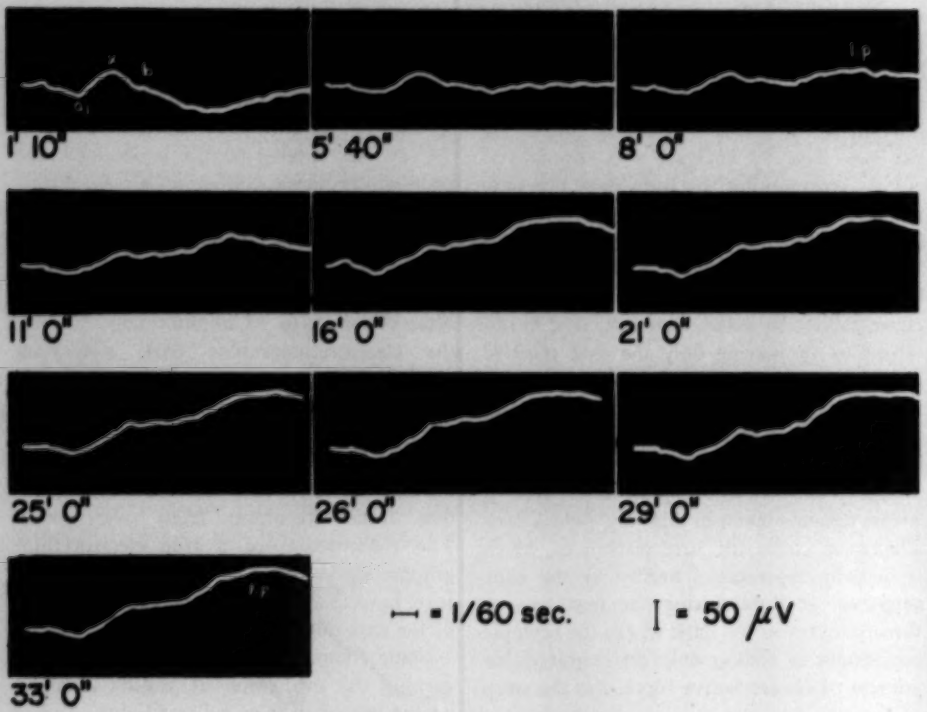

Fig. 13 (Auerbach and Burian). Behavior of electroretinogram during course of dark adaptation using Wratten filter 29 (dominant wave length at 632 mμ). There are one distinct negative wave (a_1) and three positive waves: X, b, l.p. ("late positivity").

termed accordingly these two first positive elevations obtained by the use of red test light (W29) the X-wave (for the photopic response) and the b-wave (for the scotopic response); but our b-wave is not at all identical with the second wave of the authors mentioned above. Identical with this latter wave is our third elevation ("late positivity") which had a much longer peak latency (between 120 and 145 msec.) than the actual b-wave. This is completely in keeping with the latency of the corresponding wave in Adrian's, Armington's, and Schubert and Bornschein's papers, which these authors ascribe to the scotopic mechanism and call the b-wave.

The "late positivity" (fig. 13) developed gradually during the course of dark adaptation, not being present at all at the very beginning, but showing instead a deep negative afterswing. This negativity slowly rose until it reached the level of the base line after approximately five minutes in the dark. Later it grew fast, surpassing first the b- and then the X-wave in the ninth minute of dark adaptation, and developing eventually into a huge positive wave. The barely visible a_2 never surpassed the a_1 during the whole course of dark adaptation.

2. Using another red test light (Wratten filter 70, having a spread from 650 mμ to the long wave length end of the visible spectrum and a dominant wave length of 676 mμ) we obtained electroretinographic responses of an altogether different shape (fig. 14). The fully established response from dark adapted eyes showed a first negative wave (a_1) and no a_2, X, and b; but a

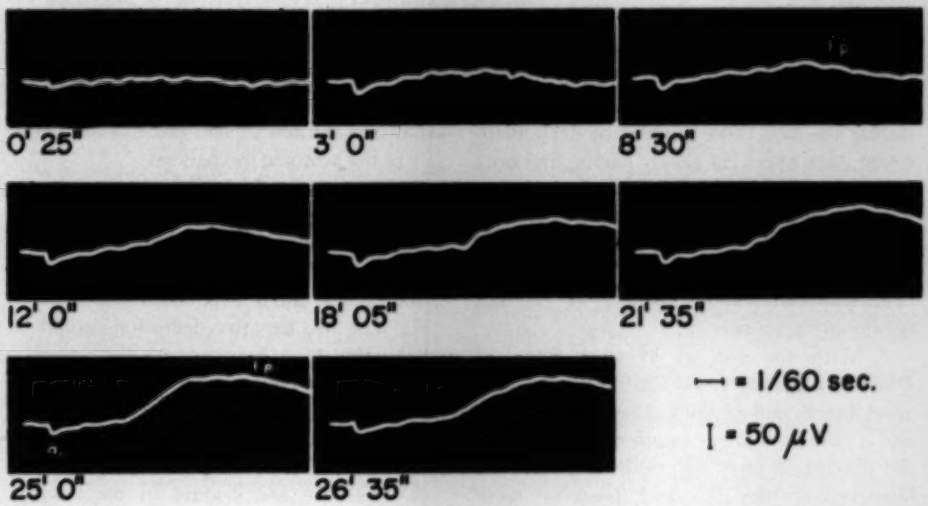

Fig. 14 (Auerbach and Burian). Similar experiment as shown in Figure 13, but using Wratten filter 70 (dominant wave length at 676 mμ). One negative wave (a_1) and one positive wave (l.p. = "late positivity").

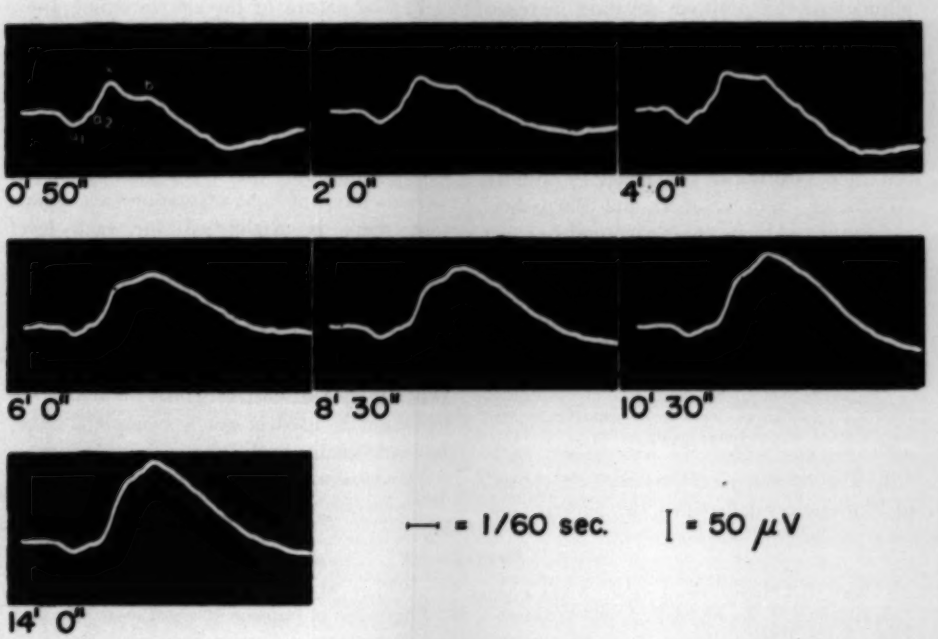

Fig. 15 (Auerbach and Burian). Similar experiment as shown in Figures 13 and 14, but using Wratten filter 22 (dominant wave length at 595 mμ). Two negative waves (a_1 and a_2), but a_2 does not surpass a_1 during dark adaptation. Two positive waves (X and b). No late positivity.

"late positivity" was present which had the same latency as the one for red test light obtained with Wratten filter 29 (125 msec.). This "late positivity" was not present at all during the first three minutes of dark adaptation, then appeared slowly having first only a very small amplitude which increased considerably during the further course of dark adaptation. The a_1-wave was after three minutes in the dark already fully established.

In a protanomalous subject we did not obtain any response.

3. With the use of Wratten filter 22 (having a spread from 550 mμ to the long wave length end of the visible spectrum and and a dominant wave length at 595 mμ) we did not obtain any "late positivity," and the latencies of the X- and b-waves were in keeping with those found with Wratten filter 29 and with white test light (fig. 15). The only differences from the response obtained with white test light were that the amplitudes of the positive elevation increased much faster, the b-wave already surpassing the X-wave after five minutes in the dark, and that a_2 did not surpass a_1 during the course of dark adaptation.

We are led by these results to believe that the "late positivities" reported by Adrian, Armington, and Schubert and Bornschein, and confirmed by us are not scotopic b-waves, but we rather think that they belong to the photopic red response. As to whether we are dealing with the response of a red color receptor (especially with Wratten 70) could not be clarified as yet.

Summary

1. The human electroretinogram consists of four distinct deflections, two negative ones (a_1 and a_2) and two positive ones (X and b).

2. The first negative (a_1) and the first positive deflection (X) appear to belong together to the photopic mechanism, the second negative (a_2) and second positive deflection (b) to the scotopic mechanism.

3. The relationship of these components of the human electroretinogram is such that at the beginning of dark adaptation $a_1 > a_2$, $X > b$, while at later stages of dark adaptation $a_2 > a_1$ and $b > X$.

4. The two negative deflections might be attributable to a response of the receptor cells; a_1 to the cones, a_2 to the rods.

5. The two positive deflections appear to be an expression of neural events, X of the photopic, b of the scotopic mechanism.

6. Thus we are dealing in the electroretinogram with two superimposed curves of slightly different latent periods, the first (a_1 X) being the photopic, the second (a_2 b) the scotopic response.

7. The nature of the electroretinographic test permits one to establish the initial threshold of the scotopic response during the photopic phase of dark adaptation. A revision of the electroretinographic dark adaptation curve is indicated.

8. Studies of the electroretinogram at various levels of light adaptation have shown that there is established for each level a characteristic electroretinogram which is the expression of an equilibrium between bleaching and resynthesis of the photopigments.

9. The third, late positive deflection appearing in the electroretinogram when red test light is used is not a scotopic b-wave, but attributable to the photopic red response.

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Discussion

Dr. Jerry H. Jacobson (New York): Does the author wish to comment upon the adaptation which was used? Was the patient in each case in complete darkness between test flashes?

Dr. Gerald E. Fonda (Short Hills, New Jersey): Do you care to comment on the application to pathology?

Dr. Edgar Auerbach (in closing): All our experiments in which we measured the electrical responses during the course of dark adaptation were performed in total darkness. In the other type of experiment where we had a constant surrounding illumination we were interested in determining the response at a steady-state.

Concerning the relationship of our results to pathologic evaluations: Of course, this was very interesting to us, but as it was first necessary to clarify the situation in normal subjects, we did not examine too many patients.

There are, as you know, two borderline conditions: the chronic simple glaucoma with a deterioration beginning at the layer of the ganglion cells which might last a very long time, even if the eye is already blind, and retinitis pigmentosa where the rods and then the cones are probably the first to suffer.

As to the former disease we obtain an almost normal electretinogram. The impulse is still conducted through the intraretinal pathways; but when a descending atrophy develops the positive potentials become distorted.

Retinitis pigmentosa represents one of the most complex and difficult problems. Tentatively, however, we believe that, in the initial stages of this condition, the receptor cell layer (a_1 or a_1 plus a_2) may display a distorted response. The proper impulse conduction depends on the normal functioning of these cells. In this condition, as long as a negative response is obtained, that is, a response of the receptor cells, even though distorted, a dis-

torted positive potential will be observed. In the more advanced stages of retinitis pigmentosa the electroretinogram is extinguished.

Perhaps it might be appropriate here to mention an observation we made in three patients.

In one of them, both eyes became amaurotic because of a temporal arteritis approximately two weeks prior to our examination. In addition he had an hypertonic fundus with many exudates and hemorrhages. The electroretinogram during dark adaptation following an adaptation to white light of 2,650 mL was normal; it had a very similar picture to normal subjects. However, an instantaneous negative deflection always appeared after an immeasurably short latent period which maintained its amplitude during the entire course of dark adaptation. It was independent of the a_1-wave.

A similar fast negative deflection, but of much greater amplitude than in the above case, was obtained in two other patients having many pigmentary products at the internal layer of the retina. The a_1, a_2, X, and b were present in one of them though distorted; in the other one they were extinguished. Again this deflection did not display any change in amplitude during dark adaptation.

We wonder whether we can account for this deflection as a localized absorption of radiant energy by those products of degeneration before it triggers the series of events which begin in the receptor cells. If that would be true, it would provide us with the possibility of observing the condition of the retinal tissue independently of the visual response.

METABOLIC INJURIES OF THE VISUAL CELL*

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Buffalo, New York

In recent years a number of chemical and physical agents have been tested as to their effectiveness upon the visual cell.[1-6] Some of these agents injure the visual cell temporarily and reversibly; others produce visual-cell effects only in lethal doses; however, a small group of agents produces irreversible effects which are selective in the sense that the damage to the visual cell concurs with the survival of other cell populations of the retina and of the organism as a whole. Agents of this kind serve, by the injury they produce, in the analysis of properties of the visual cells and of relationships between their structure and their chemical and biophysical functions.

From an experimental consideration the visual cell has much to offer for an analysis of its properties by such means. The electrical reactions of these cells to stimulation by light allows the instantaneous determination of their functional state and provides a sensitive and easy method—electroretinography—for measuring at any time after exposure to injurious agents, the extent of the functional damage which has been produced.

The progress of the effects of these agents and any recovery there from can be followed in almost a quantitative manner. Moreover, any damage which alters the histology of these cells can be detected readily due to the precise morphology of the visual cells, their orderly arrangement, their great uniformity, and their lack of growth in adult higher vertebrates.

Lastly, the very thinness of the retina and the ease with which it can be isolated as almost a whole, facilitate biochemical studies, for instance with the Warburg technique, so that three major disciplines of cellular biology can well be applied for studying the changes which have been produced experimentally.

The morphologic effects offer the most reliable basis for the evaluation of other observations and measurements. Using conventional histologic methods (fixation by Zenker-acetic acid fluid and staining by hematoxylin eosin or phloxin) two types of injury to the visual cell can be distinguished in our experimental material:

In the *first* type of visual-cell injury the abnormal histologic appearance is primarily localized in the outer portions of the cell.

* From the Roswell Park Memorial Institute.

As illustrated in Figure 1, one finds degeneration of outer and inner limbs. Early in this process the outer limbs lose the narrowness of their form and the distinctness of their borders; later, a faintly eosinophilic, almost homogenous material in the vicinity of the inner limbs and vitread and the pigment epithelium are all that one may recognize as a remnant of the outer limbs.

At this later stage the layer of the inner limbs also deviates distinctly from normal. Its normal regular appearance has been lost; the individual inner limbs differ in shape and position because of variations in the location of their ellipsoids and of variations in the thickness of their basal portion. The inner limb which signifies this type of degeneration in sections from albino rabbits has a thinned basal or intermediate portion and a broad, concave or clublike scleral end. The visual cell nuclei of the affected cells have changed little; they generally stain less intensely but their number may not be reduced when the outer and many inner limbs have degenerated beyond easy recognition.

The effect of intravenous sodium iodate is the most instructive example we found for this type of degeneration. The degenerative process of the outer portion of the visual cell is associated in this case with striking changes of the pigment epithelium. These changes include abnormalities in nuclear staining and form; cytoplasmatic swelling; increased number of epithelial cells; and complete disappearance.

Following the course of iodate poisoning by recording the electrical phenomena across the eye globe early changes become evident in the form of a marked depression of the c-wave of the electroretinogram and of the azide-response of the d-c potential.[6, 7] Disappearance or marked attenuation of the fast components of the electroretinogram (a- and b-wave) follows within a few days. The analysis of these electrophysiologic phenomena has brought forward evidence that the early electrical changes of the iodate effect depend upon the injury of the pigment epithelium.[4] The late changes result from the destruction of the sensory organelles.

It is inferred that the pathogenesis of the degeneration of the sensory organelles is related to the injury of the pigment epithelium and that direct effects of the poison upon

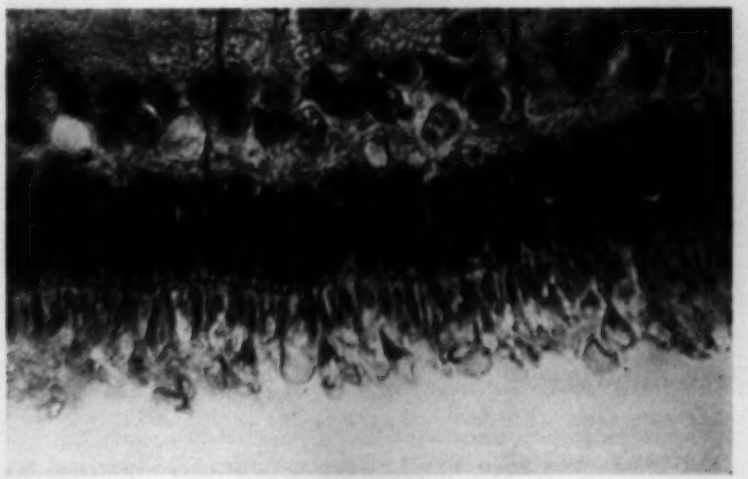

Fig. 1 (Noell). Albino rabbit. Outer layers of retina on the fourth day after a single injection of 100 mg. of sodium iodate. The outer limbs have almost completely deteriorated. The inner limbs are deformed to a varying degree. The outer nuclear layer shows no significant pathology. Hematoxylin-eosin, ×770.

the sensory organelles are of minor importance when low or moderate doses of iodate have been employed. There is evidence, both old and new, for the dependence of the rhodopsin system upon functions of the pigment epithelium. Furthermore, the pigment epithelium represents a cellular barrier between the visual cells and the choroid. This barrier may protect the visual cell as suggested by changes in the slow potentials across the eye globe and may be essential in the maintenance of retinal homeostasis. We conclude from this that an abnormal chemical condition outside of the visual cells is possibly the main cause for the loss of the viability of the sensory organelles in this type of injury.

The *second* type of visual cell injury is a sudden deterioration of all cell organelles in response to poisoning by chemical or physical agents. The prototype of this injury is produced by intravenous iodoacetate in doses which affect only the most sensitive fraction of the visual cell population.

A few hours after the administration of the poison, the visual cell layer displays pyknosis, and swelling and deterioration of the sensory organelles. This cell death is selective in the sense that no significant histologic changes of other retinal structures including the pigment epithelium are present as long as the poisoning is not excessive. The end-result of this injury is the complete disappearance of the affected visual cells with the preservation of the other retinal layers (fig. 2). The outer retinal border is then formed by the outer limiting membrane in direct contact with the pigment epithelium.

Because of its acuteness this injury must be considered a direct effect of the poison upon the metabolism of the visual cell. The selective manner in which this effect is produced points to one or more exceptional metabolic properties of the visual cell which renders it specifically susceptible to this poison. The injury is, therefore, called "metabolic."

The essential characteristics of this meta-

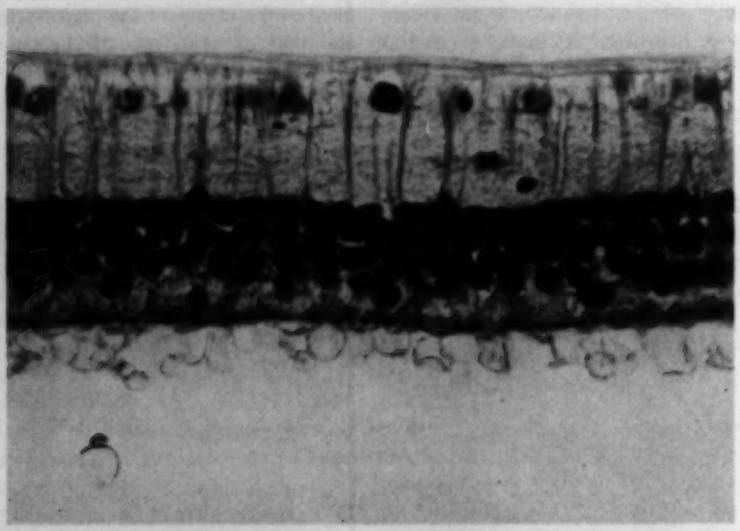

Fig. 2 (Noell). Albino rabbit. Retina two and a half months after a single injection of 20 mg. per kg. bodyweight of iodoacetate acid. The section is from a small area of visual cell degeneration in the ventral half of the retina, close to the central region. The visual cells have completely disappeared; the inner layers are well preserved. There is no retinal distortion and practically no gliosis. Looplike glial fibers extend from and through the external limiting membrane toward the pigment epithelium. The separation of the retina from the pigment epithelium is artificial and has occurred during fixation. Hematoxylin-eosin, ×620.

bolic injury are: (1) The almost simultaneous histologic involvement of all portions of the visual cell and (2) the preferential involvement of the rod cells to the extent that the effect of iodoacetate in the monkey shows the same distribution as in hereditary degeneration of the neuroepithelium (retinitis pigmentosa).

Two other procedures, quite different from poisoning by iodoacetate, have been found also to produce metabolic injury of the visual cells in adult animals: high intensity X-radiation[5] and oxygen poisoning.[6] These additional examples for selective visual cell injury widen the scope of the experimental efforts for the analysis of visual cell properties and also facilitate these efforts by enabling one to search for common factors which determine the death of the visual cell in these cases. In addition, these effects provide the opportunity to utilize the visual cell as a tool of cellular physiology and to quantitate, by visual cell reactions, basic biologic phenomena of the effect of these agents, findings of great medical interest. In the following, the most recently studied effect of high oxygen concentration shall be described in order to illustrate these points.

The study of the effects of oxygen upon adult visual cells was stimulated by Gershman's hypothesis that the primary mechanisms of action of high oxygen pressure are in part the same as those of ionizing radiation.[8] Since the visual cells of rabbits and other species are injured by high intensity X-radiation in a selective manner it was thought worthwhile to test this hypothesis by determining the sensitivity of the visual cells to high oxygen pressures.

To this end, albino rabbits of two to three kg. body weight were exposed to oxygen pressures ranging from 350 mm. Hg at ambient pressure to 90 lb./sq. inch. Carbon dioxide was absorbed by soda lime and its chamber concentration kept close to a level less than 0.1 percent. The chamber temperature ranged between 70 to 80°F. The animals were generally unanesthetized. Their electroretinograms were recorded either continuously when fast effects were expected or intermittently at pressures which produce their effects slowly. Reversible and irreversible changes were observed almost throughout the pressure range studied. Irreversible effects were most extensive after exposure to high oxygen concentrations at ambient pressure.

The histology of this effect, 16 days after exposure to 100-percent oxygen for 40 hours, is illustrated by Figure 3. The great majority of the visual cells of this section have disappeared. The outer nuclear layer is reduced from normally four or five rows to one or two rows of nuclei. Outer limbs are not recognizable, whereas some stumps of inner limbs still project through the external limiting membrane from visual cells which have survived.

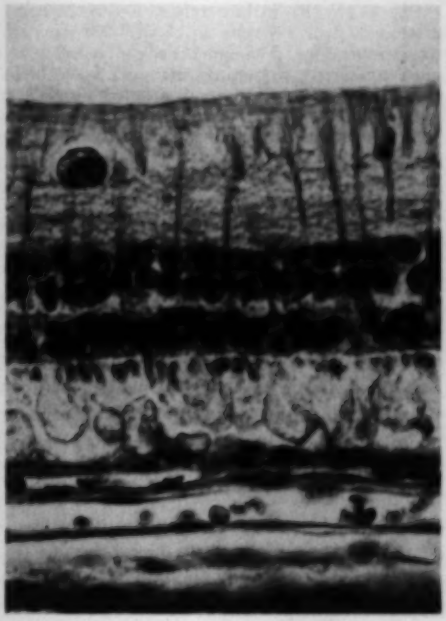

Fig. 3 (Noell). Albino rabbit. Retina 16 days after exposure to 100-percent oxygen for 40 hours. The outer nuclear layer is reduced to one or two rows of nuclei. The outer limbs have completely deteriorated. The majority of the inner limbs are also unrecognizable; other inner limbs have changed to short stumps. The pigment epithelium shows an increased number of cells. The inner layers are preserved. Hematoxylin-eosin, ×630.

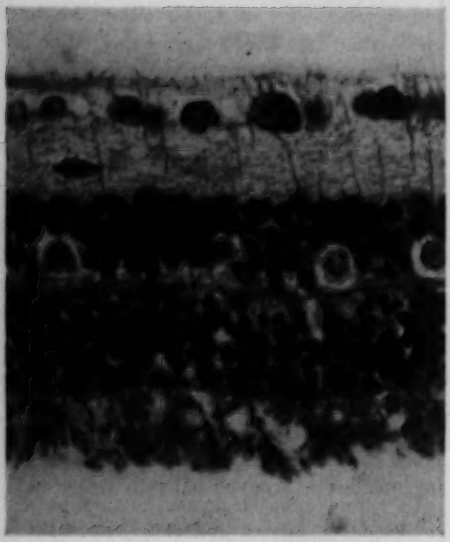

Fig. 4 (Noell). Albino rabbit. Retina 24 hours after a 30-hour exposure to about 1,000 mm. Hg oxygen pressure. The outer nuclear layer is almost completely pyknotic. Outer and inner limbs are markedly deformed and disintegrating. The inner layers are preserved. Mueller cells seem to move into the pyknotic outer nuclear layer. Hematoxylin-eosin, ×630.

An earlier stage of this histologic effect is illustrated by Figure 4. It shows the typical picture of the metabolic injury of the visual cells between 10 and 24 hours after the onset of electroretinographic changes during exposure to high oxygen pressures.

Almost the whole outer nuclear layer is pyknotic and the sensory organelles have completely deteriorated. The number of elements in the outer nuclear layer is less than the number of nuclei which one normally finds in such section. Consequently autolysis of the missing nuclei must have already occurred at this stage. They probably were the first to become pyknotic.

The histologic effect of oxygen poisoning shows a characteristic distribution over the retina which is practically the same as after poisoning with iodoacetate. In vertical sections through the rabbit's eye (fig. 5) the most sensitive visual cells are located in the ventral half of the retina close to the central region. With prolonged exposure to oxygen poisoning, death of the visual cell spreads from this region first over a greater area of the ventral half and then over the other parts of the retina, but visual cells close to the ora serrata and to the optic nerve generally survived. At the present time we are still unable to decide whether this pattern of distribution is related to local differences in choroidal circulation or whether it manifests true metabolic differences within the visual-cell population. It is hoped that quantitative determination of visual cell sensitivity to X-radiation in the different retinal areas may decide this question.

The electroretinographic change during exposure to high concentrations of oxygen at ambient pressure mainly consists of an attenuation of all waves. In order to quantitate this effect, the amplitude of the b-wave at different stimulus intensities was measured and compared with its control amplitude under the same conditions of adaptation. A significant attenuation of the b-wave was manifest in about 70 percent of all animals tested at the 25th hour of exposure to 100-percent oxygen. At 36 hours of exposure practically all animals were affected and the b-wave had declined in the average to less than 30 percent of its control size. These changes were reversible to some extent when the animal was allowed to recover in normal atmosphere. In the average, recovery occurred for those changes which had developed during the last six to 10 hours of exposure to 100-percent O_2. These data are put forward merely to indicate the great value of the electrical methods in quantitive determinations of an injurious effect.

Irreversible effects of oxygen poisoning upon the visual cell were observed with oxygen concentration as low as 60 percent at ambient pressure. Seven out of 12 animals exposed to this concentration for seven days showed visual cell death over small to large retinal areas. Some pulmonary involvement (edema, hemorrhage) was almost always as-

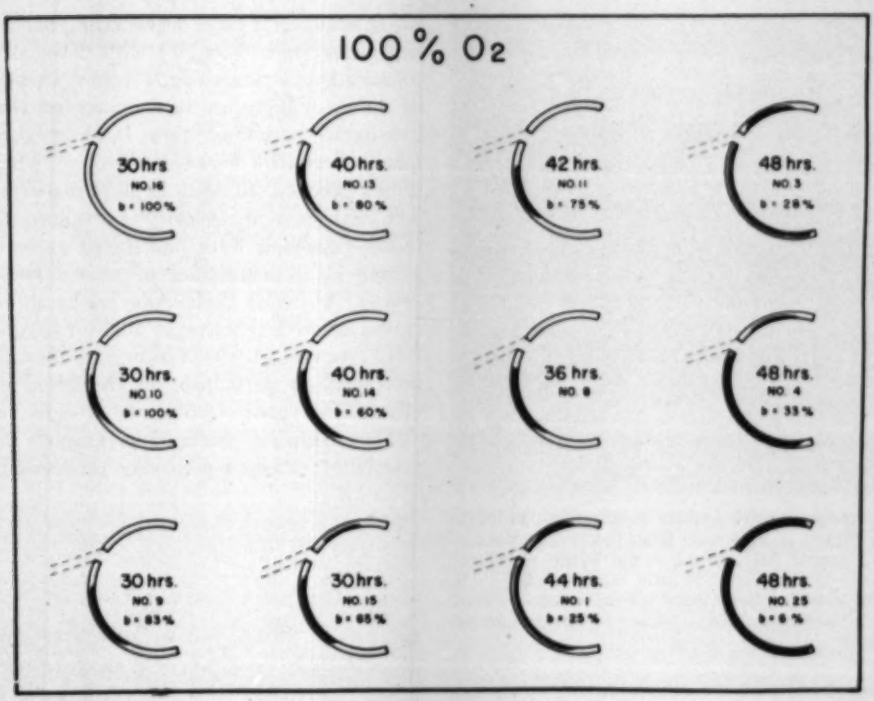

Fig. 5 (Noell). Schematic representation of the extent of the visual cell degeneration in vertical sections through the eye after 100-percent oxygen. The drawings are based on the extent of damage in the outer nuclear layer which in the diagram is represented by the space between the two semicircular lines. Black areas indicate the dead visual cells (missing or pyknotic nuclei). At least a few nuclei have survived in every affected area of each retina. The data for b-wave amplitude were obtained prior to the preparation for histology, that is five to 18 days after the end of exposure. Exposures to O_2 lasted for 30 to 48 hours as indicated.

sociated with the retinal effect after exposure to oxygen at ambient pressure. It was very rarely, however, of such degree that the animal was unable to survive the change to normal atmosphere, not even in cases in which the great majority of the visual cells had been affected irreversibly.

The pulmonary involvement probably accounts for the fact that despite the high sensitivity of the visual cell to oxygen it proved impossible to affect all cells irreversibly by prolonged exposure or to affect in any retinal area the cells to the same extent as with iodoacetate (fig. 2). At least parts of one row of the visual cell nuclei survived in almost all cases. Furthermore, among the surviving cells were apparently all those normally present which have a cone cell-like appearance as evidenced by a large nucleus and by a fine distribution of the nuclear chromatin. It is, therefore, concluded that the cone cells are more resistant to oxygen poisoning than the rod cells in the same manner as they tend to resist the effect of iodoacetate and X-radiation.[2, 5]

The surviving visual cells after oxygen poisoning show normal nuclei but markedly disformed sensory organelles (figs. 3, 6, and 7). This abnormality of the outer portion of the visual cell can have the following two causes:

First, it may represent an atrophic process which is initiated by the degeneration and disappearance of the sensory organelles in

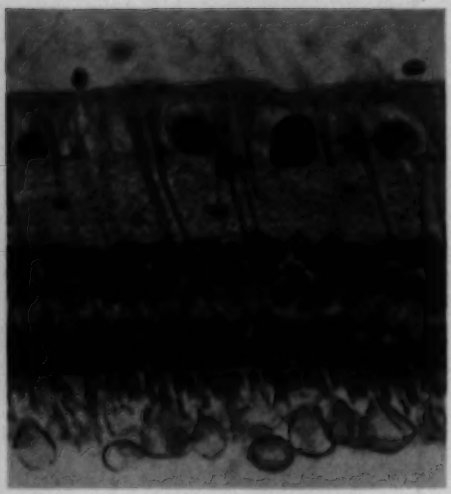

Fig. 6 (Noell). Albino rabbit. Same retina as in Figure 3. Section is from the midperiphery of the ventral half of the retina where visual cell death is less extensive than more centrally. The still surviving inner limbs are deformed in a similar manner as after iodate. Hematoxylin-eosin, ×630.

the vicinity of the surviving one. One can easily imagine that the surviving outer and inner limbs are exposed to increased mechanical stress when support by adjoining elements vanishes due to degeneration. Single limbs can obviously not effectively oppose the stress of the intraocular pressure. It has been outlined previously that this mechanical stress upon the surviving cones of the parafoveal and peripheral region in cases of selective rod cell degeneration produces their shortening and broadening to the extent that the spaces normally occupied by the rods are refilled.[4] A similar atrophic process may account for the shortening and broadening of the inner limbs after poisoning by oxygen. The same limb changes are observed also after iodoacetate when only a fraction of the visual cells has degenerated in any one area.

The *second* cause for the isolated degeneration of the sensory organelles may be similar to the one outlined for the effect of iodate. In fact, there is a great similarity in the appearance of these degenerating sensory organelles (figs. 6 and 7) with those after iodate administration (fig. 1). Such changes of the inner limbs are rarely observed after iodoacetate poisoning; they, however, commonly occur after X-radiation in doses which do not produce cell death in all retinal areas. The changes of the sensory organelles after iodate poisoning were interpreted as to be caused by a disturbance of retinal homeostasis. A similar explanation seems appropriate for oxygen poisoning and X-radiation. Both types of visual-cell injury are thought, therefore, to participate in the histologic effects of oxygen poisoning and X-radiation.

The following observations support this possibility: Oxygen poisoning produced ir-

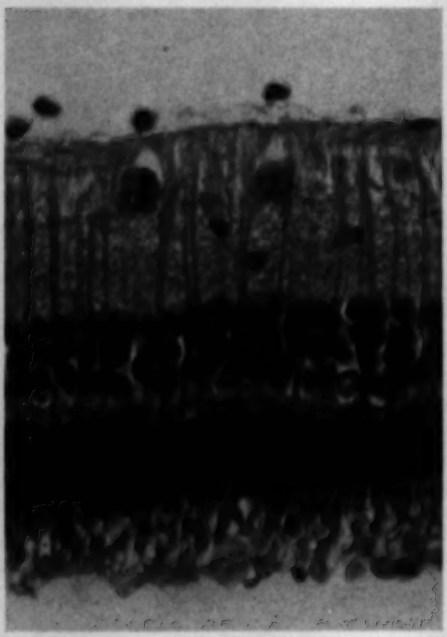

Fig. 7 (Noell). Albino rabbit. Retina five days after exposure to about 65-percent O_2 for 100 hours. The section is from an area where visual-cell death does not involve more than one or two rows of nuclei. There is, however, a marked deterioration of the outer and inner limbs. Note the inflammatory cells close to the internal limiting membrane and at the border of the outer limb layer. Hematoxylin-eosin, ×630.

ritation of the uveal structures in many of the animals, especially in those which were exposed to 60 to 85-percent O_2 for four days (80-percent O_2) to seven days (60-percent O_2).

Edema of the iris and especially of the ciliary body was frequently found histologically for several days after oxygen poisoning. Increased coloration of the intraocular fluids and of the iris by intravenous trypan blue administered at the end of exposure occurred in all animals tested in which the electroretinogram was affected. Abnormal protein precipitation was often present around the ciliary processes and within the vitreous; monocytes had collected close to the internal limiting membrane.

Large and small, single or multiple rosette formations which are typical for the iodate effect were revealed in the routine sections from 25 percent of the animals exposed to high oxygen at ambient pressure. They were mostly localized at the ora serrata (fig. 8). The same changes, but generally much more excessive (with the exception of rosette formations), were observed after X-radiation of the eye. The pigment epithelium after O_2 poisoning was only slightly affected histologically; there was slight cytoplasmatic swelling and some increased cellularity.

The finding of the two types of visual cell injury in both X-radiation and oxygen poisoning further supports the supposed similarity of these effects. The type-1 injury deserves special recognition in quantitative determinations of these effects by means of electroretinography. The same reduction in a- and b-wave finally is produced by the isolated degeneration of the sensory organelles as by the death of the cells. However, quantitative relationships for the biologic effects of these agents should best be derived from measurements of their direct action upon the visual cells.

As yet no conclusions have been reached as to the properties of the visual cell upon which its selective sensitivity depends. Noell and Pawel (unpublished) measured O_2 consumption and glycolysis of retinas which had been almost completely deprived of their visual cell population by intravenous administration of iodocetate three weeks prior to the chemical determinations (table 1). In comparison with normal controls it was found that retinas without visual cells have less glycolysis than normal retinas so that one may conclude that retinal glycolysis is especially a property of the visual cells. High glycolysis of the visual cell has been assumed previously for the explanation of the selective effect of iodoacetate.

Warburg measurements on retinas re-

Fig. 8 (Noell). Albino rabbit. Two large rosettes close to the ora serrata. The rabbit was exposed for four and one-half days to 70-percent to 80-percent oxygen. The eye was removed 10 hours after the end of exposure (the rosette formation must have occurred during exposure). The b-wave was reduced to 35 percent of the control amplitude. Visual-cell death did not extend to ora serrata. Hematoxylin-eosin, ×200.

TABLE 1

CONSUMPTION AND GLYCOLYSIS OF RETINAS DEPRIVED OF VISUAL CELLS

	Q_{O_2}	$Q_{G^{O_2}}$	$Q_{G^{N_2}}$
	(mg. dry weight)		
Normal retinas	21.2	33.2	69.4
Retinas without visual cells	26.3	14.5	46.2

TABLE 2

CHEMICAL MEASUREMENTS

	% Change		
	Q_{O_2}	$Q_{G^{O_2}}$	$Q_{G^{N_2}}$
Iodoacetate			
0.5 hr.	−30	−78	—
4.0 hr.	−12	−55	−55
24.0 hr.	−42	−41	−40
X-radiation			
0.5 hr.	−34	−20	−23
4.0 hr.	−42	+7	+3
24.0 hr.	−50	−9	−8

moved at different times after the administration of iodoacetate in doses which produce the death of the great majority of the visual cells further showed that retinal glycolysis is severely impaired immediately after the irreversibility effective (*second*) injection of the poison. Whether this alone or in conjunction with the reduction in O_2 consumption suffices to produce cell death we do not know.

A similar study was performed after X-radiation of the retina which produced the same acute and irreversible failure of the visual cells. The electroretinogram tested prior to the removal of the retina was as abolished as after iodoacetate poisoning. With both agents (in the doses used) the histologic signs of visual cell death develop about six to eight hours after the disappearance of the electroretinogram. Table 2 shows that despite the similarities in the physiologic and histologic effects, the chemical measurements differ appreciably.

Glycolysis is obviously not involved to any great degree in the effects of X-radiation upon the retina. It is also very questionable that the measured reduction in O_2 consumption, which is of the same magnitude as acutely after iodoacetate, is the cause of the death of the visual cell population. Much more extensive biochemical studies, measuring other metabolic functions, are obviously needed to explain the physiologic effects. It is hoped that such studies will finally succeed in defining the primary mechanisms of action of these and other agents upon the visual cell and that they will yield a better understanding of the normal functions of this remarkable cell.

Roswell Park Memorial Institute.

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DISCUSSION

DR. ZACHARIAS DISCHE (New York): Dr. Noell's experiments are, I think, of great interest, not only from the point of view of the ophthalmologist, but also from a more general biochemical point of view, first of all because of the great similarity of the effects of three such different factors as intravenous iodoacetate, oxygen poisoning, and X-radiation. In all such selective effects by dif-

ferent agents, we can hope, on one hand, that we can learn something about the chemical structure of the affected cells; and, on the other hand, if we succeed in localizing these effects in such a cell, we can hope to learn something about their mechanism.

Dr. Noell assumes, apparently, that the iodoacetate effect is purely metabolic, that it is due to an inhibition of glycolysis which is one of the most characteristic and general effects of this compound. But, I think it would also be worthwhile to consider whether the iodoacetate does not liberate some iodine when it is injected into the body.

We made some experiments years ago; and we found that if we add iodacetate to hemolyzed blood and incubate at 37 degrees we always get some liberation of iodine. Iodine is a strong oxidizing agent, and it would be possible that its production is particularly high in the visual cells, due to specific local conditions. It is more difficult, I think, to visualize a direct selective metabolic effect against glycolysis.

I do not know of any experiments which would indicate a higher sensitivity of the rods as compared with other tissues against iodoacetate.

The striking parallelism between the effects of X rays and high oxygen tension on the other hand would indicate that oxidizing radicals are formed by these oxidizing agents; and that they oxidize some specific structures in the cells as suggested by R. Gershman and her associates.

There are two classes of compounds in cells which are generally considered as particularly sensitive to oxidizing agents; namely, SH-groups on proteins and certain unsaturated fatty acids. As was particularly shown by the experiments of Meade and Pennyrod, a saturated acid in lipids can also be attacked by high-oxygen tension.

A certain similarity of the mechanism of the effects of X rays and oxygen on the unsaturated acids and living cells can be deduced from the similarity of the protective effects of certain antioxidants in both these systems (Gershman et al.).

All these would rather suggest that we should look whether certain phospholipids and particularly lipoproteins are not the point of attack of these strongly oxidizing agents.

It has been found that the outer limbs of the rod contain formations which are interpreted as combination of lipids with proteins and which appear as very prominent structures. I think that these elements may have something to do with the specific function. Of course, any damage to such structures would have a very considerable effect on survival of such a function of these elements.

Dr. David G. Cogan (Boston, Massachusetts): I would like to ask Dr. Wald if the vitamin A—rhodopsin cycle is poisoned by iodocectate.

Dr. George Wald (Cambridge, Massachusetts): Dr. Noell and I have talked about this before.

Actually, iodoacetate does not poison this system; yet it is a sulfhydryl system, and it is poisoned by more powerful and more specific sulfhydryl reagents, such as para-chloromercuribenzoate (PCMB).

I think, with Dr. Dische, that the array of treatments which Dr. Noell has told us of in these very interesting experiments does make one think, throughout, of oxidations of easily oxidized groups.

Sulfhydryl groups are among the first things one would think of. The problem is not to draw too specific or limited consequences from this possibility. Roughly half the enzymes known in biochemistry are sulfhydryl enzymes. These offer a multitude of points of attack; so that one might get quite different results for every different situation which one tackles. I think that no one kind of effect is to be looked for from this source.

The unsaturated fatty acids, of course, are very important, too. In the outer segments of the rods which have been analyzed, about 35 percent of the dry weight is a mixture of lipids, and these play a very important part in the microstructure. Their unsaturated fatty acids are easily oxidized, and sometimes promote the oxidation of other substances catalytically, as in the lipoxidase system.

There is a third thought which I would like to suggest to Dr. Noell. That is that one of the real surprises in recent years has been the realization that, in a rod, the visual pigment (the rhodopsin, for example) is not merely something which happens to be there, but that a large part of the structure of a rod is rhodopsin; and, when something attacks the rhodopsin, the rod outer segment will visibly go to pieces. The outer segment and its rhodopsin are in large part the same thing; something of the order of 60 percent of the dry weight of a frog rod is rhodopsin.

That would not be true of a cone. A cone has very little visual pigment, and the visual pigment must play a very small role in its structure. So, when we see situations (pathologic and experimental) in which the rods are much more susceptible than the cones, one wonders a little whether one is not attacking the visual pigment itself.

Dr. Zacharias Dische (New York): The suggestion, Dr. Wald, that the SH-groups are affected, of course, comes up immediately in our minds when we think of the point of attack of oxidizing agents. It is striking, however, how little affected these SH-groups are in irradiation injuries of such structures as the lens. In investigations of Pirie, for example, only after many weeks was it possible to find a certain decrease in SH-groups of four SH enzymes in the lens.

On the other hand, the SH-groups of the lens proteins themselves, which are extremely active against many other oxidizing agents, and very labile, do not react at all in the first few weeks after irradiation.

Therefore, I think that, although it has been shown that irradiation affected SH-groups of enzymes and proteins in vitro, this has not been shown very clearly in the case of living cells. There may be a very great difference between the latter and in vitro systems.

On the other hand, experiments of Penrod and Meade are very suggestive, in the sense that the unsaturated fatty acids are perhaps still more sensitive, and react much more generally to X rays and high-tension oxygen.

Dr. David G. Cogan (Boston, Massachusetts): The terminal half of the rod contains a periodic acid-Schiff positive substance which is easy to stain with the standard technique. If Dr. Wald is correct, and the effect is directly on this polysaccharide, it would be interesting to know if the earliest morphologic change were not a change in the Schiff-positive material. I wonder if Dr. Noell happens to have observed this in his threshold or subthreshold doses of iodoacetate or oxygen poisoning?

Dr. Werner K. Noell (in closing): I am very grateful to Dr. Dische and Dr. Wald for their suggestions. They gave me much to think about; and we, certainly, will follow up the points they made.

Let me first say that I left myself a way out by stating that we do not know whether the effect of iodoacetate on visual cell glycolysis suffices to produce cell death. Inhibition of glycolysis is definitely involved but other effects of iodoacetate may render this inhibition fatal and these effects may be even more decisive than the effect upon glycolysis.

There are three essential phenomena of the "metabolic injury" which one must consider in relation to the mechanism of action of our agents:

First, as was pointed out, the cone cells survive. The explanation of our effects must, therefore, provide a reasonable suggestion for the difference in rod and cone cell susceptibility.

Second, our "metabolic injury" is produced acutely; iodoacetate, for instance, produces a measurable and very rapidly progressing effect on the electroretinogram within 20 or 30 seconds after intravenous injection. I think that this limits our possibilities as to the mechanisms of action of these agents.

Thirdly, the "metabolic injury" involves almost simultaneously all organelles of the rod cell. This is quite different from our type 1 injury of the visual cell in which the outer and inner limbs degenerate slowly. In the "metabolic injury," the nucleus, the inner limb, and the outer limb show signs of severe damage at the same time as if the agents had hit a vital point within the cell. What that vital point is, in a chemical or structural sense, I do not know.

In short, I do not think that the "metabolic injury" can be explained by primary effects upon the rhodopsin system or upon any other component specific for the outer limb. Effects on the outer limbs are compatible with cell life; they do not produce cell death as does the "metabolic injury." For instance, practically all outer limbs may be destroyed by injection of sodium iodate, but the visual cell nuclei may survive for one or two more weeks after the outer limbs have virtually disappeared.

We looked at many retinas which were removed one-half hour after the injection of iodoacetate for biochemical studies. Their color was nice, just as pink as it should be; they bleached as nicely and rapidly as the controls. So I assume rhodopsin was still there despite the fact that the poison had "paralyzed" the visual cells, that is, had rendered them unresponsive to stimulation by light.

In answer to your question, Dr. Cogan, we have no evidence suggesting a threshold change in light sensitivity during the acute action of iodoacetate as long as all the visual cells still produce one component of the electroretinogram (for instance, during the short phase after iodoacetic-acid poisoning in the rabbit when the b-wave has disappeared and the a-wave is still preserved). We observe changes later, when the population of reacting visual cells has decreased.

AN EXPERIMENTAL STUDY OF THE ELECTRORETINOGRAM*

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INTRODUCTION

Our purpose in conducting this investigation has been to study the electroretinographic changes during dark adaptation in the rabbit.

The functional units of the electroretinogram complex have been suggested previously by several physiologic experiments. Among them is the work of Granit[1] demonstrating the progressive disappearance of the P_1, P_2, and P_3 waves under anesthesia. Granit also hypothesized that the positive electroretinogram voltage arose from the bipolar cells. More recently there has been the extensive investigation of Noell[2] in which he studied the electroretinogram following the intravenous administration of certain drugs to animals.

Also an analysis of the composition makeup of the electroretinogram has been attempted in dark-adaptation experiments and in studies utilizing colored lights as stimuli. In 1945 Adrian[3] pointed out the existence of two positive waves in the rod-cone retina but obtained only one positive wave in the rabbit and other predominantly rod-retinas. He believed the first positive peak of an electroretinogram wave was an electrical manifestation of photopic vision. The later positive peak signified activity involved in scotopic vision. In 1952 Armington, Johnson, and Riggs[4] described two negative waves. Bartley[5] suggested that the *a-wave* is a receptor cell response. In 1954 Burian[6] related the second positive wave to dark adaptation and said that the dual positive wave revealed both photopic and scotopic elements. Auerbach and Burian now postulate a direct relationship between the double negative wave of Riggs and the double positive wave of Adrian. Their contention is that a_1 fires the X or first positive wave and is a photopic phenomenon and that a_2 fires the b or second positive wave which is scotopic in nature.

Our approach was to study the normal rabbit retina with three techniques: (1) Fundus photography, (2) histologic sections, and (3) electroretinograms during dark adaptation.

Following the study of normal eyes it was our intent to produce various changes in the retina with three different poisons: (1) Alloxan, (2) Neotetrazolium, and (3) Dithizone.

Then we had hoped to investigate the damaged retina with the same techniques.

PROCEDURE AND RESULTS

For our experimental subjects we selected 25 adult albino rabbits and two gray rabbits with pigmented retinas. Our photographer and technical adviser was Lee Allen.

With the exception of topical pontocaine no anesthesia was used to record electroretinograms. This was to avoid any possible effect of general anesthesia upon the neural response. An attempt was made to make the rabbit as comfortable as possible to avoid movement artefact in the recording. The animal was restrained with foam rubber and placed on a table which had a light tight cover. Modified ear rings grounded the animal. The pupil was dilated by atropine. A special light weight lid retractor with a polyethylene irrigator was constructed of stainless steel to prevent the lids from wiping off the corneal electrode which was made of number-38 fine silver wire coated with silver conducting paint. This electrode proved so light and flexible that electrical artefact from

* From the Department of Ophthalmology, College of Medicine, State University of Iowa. This research was supported by a grant-in-aid from the Institute of Neurologic Diseases and Blindness, U. S. Public Health Service, and the Arnold Reuben Fight for Sight Fund of the National Council to Combat Blindness, Inc.

eye movement was reduced to a very low level. Construction details of these electrodes will appear in a future article. The "inactive" electrode was placed on the skin of the supra-orbital ridge.

The sequence of events in obtaining the dark-adapted electroretinogram was as follows. The period of light adaptation lasted 10 minutes. The light was a G.E. 150-watt reflector spot lamp with a five-inch water filter. The animal and stimulating lamps were enclosed in a light-proof ventilated testing table. When the base line had settled, the flash tube was energized. This tube was a GR type 648 activated by a 400-volt discharge from a 10 microfared condenser. Both light sources were 17 inches from the eye. The initial flash could usually be presented in 0.5 to 3.0 seconds after the beginning of dark adaptation though the response to this flash was too small to be measured in many cases. Subsequent flashes were presented at the following intervals or as near them as the appearance of a steady base line permitted: every 30 seconds for the first five minutes; every minute for the next five minutes; and every five minutes thereafter. Responses were amplified by an Offner type 142 amplifier with time constant controls set for about one second.

No attempt was made to make histologic sections on the eyes of rabbits given Dithizone. These rabbits either died at the time of injection from massive congestion of the heart by precipitated Dithizone or developed large swellings at the site of injection and continued to produce normal electroretinograms. We cannot explain our failure in administering this drug. Large doses of Neotetrazolium, 6.0 cc., produced no changes in the electroretinograms. We hope to repeat this portion of our study administering one-half cc. per day as suggested by Dr. Goodwin Breinan.

Sections from retinas of the alloxan series appeared normal, although their electroretinograms were grossly abnormal. The longest survival period after injection in this group was 11 days, which is apparently too short a time in which to produce observable neural degeneration.

Histologic sections of the normal rabbit retina showed it to be a predominantly rod-retina.

The fundus photographs were somewhat more informative, as may be seen in Figure 1.

The electroretinograms of normal rabbits generated during the course of dark adaptation revealed several changes. Figure

Fig. 1 (Davis and Arnott). Fundus photographs before and after alloxan. The character of the choroidal vessels is apparent in the normal retina of the rabbit on the left. On the right is shown the fundus of the same rabbit following the administration of alloxan. There is considerable diminution in the number and caliber of the choroidal vessels.

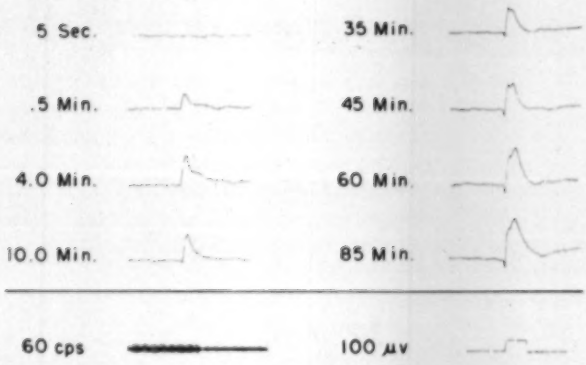

Fig. 2 (Davis and Arnott). The normal growth of the electroretinogram during dark adaptation. (Rabbit.)

2 depicts the normal growth of the electroretinogram during dark adaptation.

The following generalizations are based on our observations. The positive deflection increases in amplitude up to 30 or 60 minutes. At from five to 20 minutes a second positive hump appears on the descending slope of the first positive wave. This second positive wave grows faster in magnitude than the first. The two positive deflections are about equal after one-half hour in the dark. The later positive hump then exceeds the first. Stability is then reached with this relationship constant. The amplitude is about 300 microvolts. The negative deflection is apparent in from 15 to 30 minutes. It slowly increases in amplitude for 20 to 50 minutes after its appearance and then is constant.

Figure 3 is a larger photograph of a normal dark-adapted tracing which shows the time relations between the flash and the various parts of the electroretinogram wave. At no time did a double negative wave appear in our tracings. Dr. Burian, co-author of the previous paper concerning dual negative deflections, has examined our recordings and agrees that the rabbit's negative response to an intense flash of light is single in nature. It would seem, then, that the double positive b-wave is not related in all respects to the double a-wave. Our single a-wave was to be expected. Histologically, the rabbit is primarily a rod animal. Discrimination color tests have shown the rabbit to be color blind. Previous investigators found only a single a-wave in the rabbit. What, then, is the physiologic explanation for a single a-wave with its subsequent dual positive b-wave and why does the relative magnitude of the two positive deflections change with dark adaptation in a scotopic animal? If the rabbit possesses functional rods and cones, a dual a-wave might be expected.

Many of our alloxan rabbits died without developing abnormalities in the electroretinogram. However, some responded to light with only a positive wave and others with only a negative wave. Figure 4 may be con-

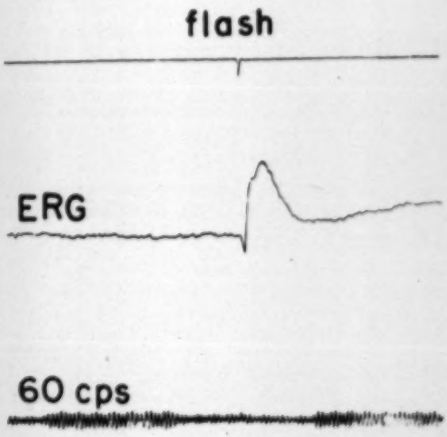

Fig. 3 (Davis and Arnott). Normal dark-adapted electroretinogram. (Rabbit, 80-minutes dark adaptation.)

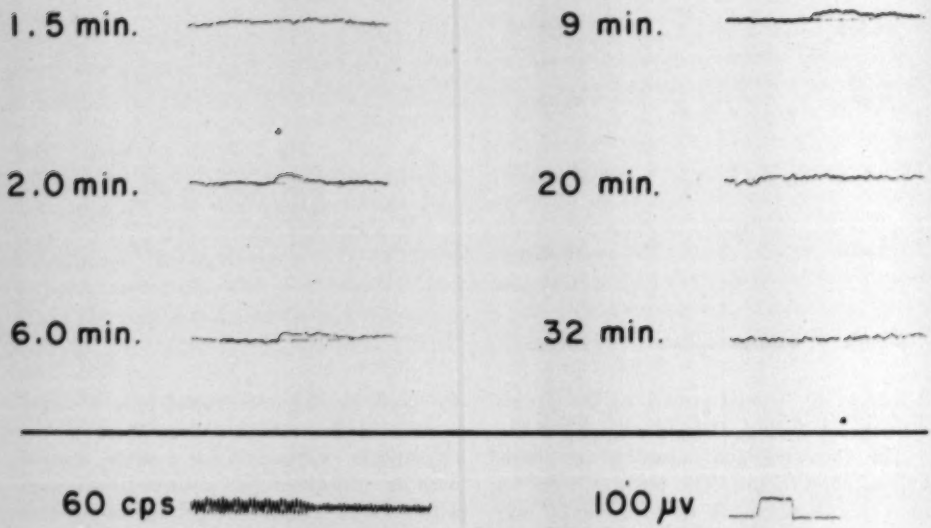

Fig. 4 (Davis and Arnott). Positive deflection during administration of alloxan. (Rabbit, 13A, six days after alloxan.)

veniently referred to as the alloxan-positive type.

A positive wave of reduced amplitude appears a few minutes later than the normal positive wave. Its growth is small; after reaching a maximum at five minutes it declines to zero at about 20 minutes. No negative wave appears at any time.

This could be merely a normally shaped response so reduced in amplitude that only the positive deflection is perceived. Disappearance of this wave at 20 minutes is diffi-

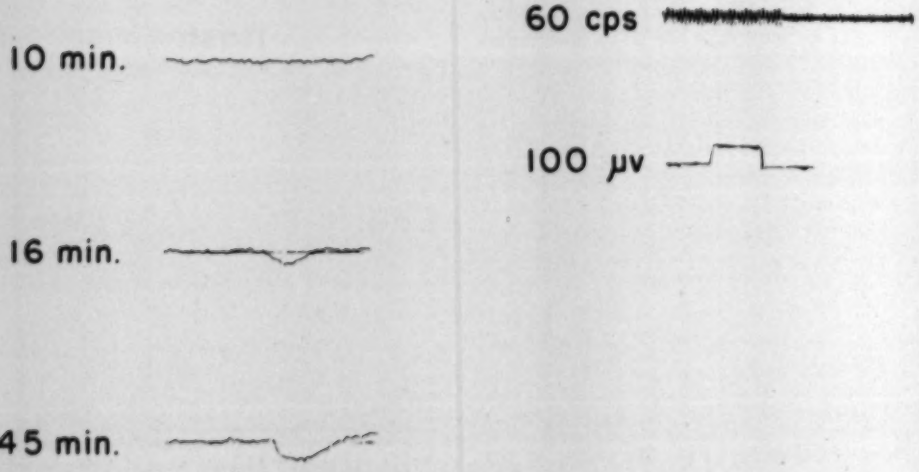

Fig. 5 (Davis and Arnott). Negative deflection following administration of alloxan. (Rabbit 14, 11 days after alloxan.)

cult to explain. One guess is that vasoconstriction occurs in the dark-adapted retina which normally has little measurable effect but with the already present diminished blood supply of the alloxan rabbit we observe complete loss of measurable electrical response. A light initiated vasodilatory reflex might explain other experimental observations. More often than not in our recordings b-wave amplitudes were not quite as great after prolonged dark adaptation as they were at 20 or 30 minutes. Austin Riesen[7] found retinal circulation impaired and vision irreversibly damaged in animals raised in total darkness.

Figure 5 is a photograph of the alloxan-negative type recordings. A negative wave appears at the same time as in normal records. It is similar in amplitude. Its duration is much greater approximating the duration of the entire normal electroretinogram. Possibly the receptor cells in these rabbits were not damaged as severely as were other retinal layers. Our results here appear similar to Granit's observations concerning P_3.

Conclusions

Electroretinograms from the rabbit, which has usually been considered a scotopic animal, show, after prolonged dark adaptation, a single a-wave but a dual b-wave. Explanations of the dual positive b-waves originating from dual negative waves appear inadequate.

The positive wave of some rabbits with a diminished blood supply can no longer be elicited after prolonged dark adaptation. Possibly a light-initiated vasodilatory effect exists in the eye. Loss of the positive wave after 10 to 20 minutes of darkness may be the result of reduced circulation resulting from prolonged darkness and severe alloxan poisoning.

University Hospitals.

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Discussion

Dr. Albert M. Potts (Cleveland, Ohio): This paper is of interest to all of us, because it represents another attempt similar to some of the preceding ones to analyze the electroretinogram into its components on an anatomic or on a physiologic basis. However, there are some cautions which should be given.

First, in regard to the use of the rabbit as a predominantly rod animal: On a statistical basis, the human is also a predominantly rod animal; there are other physiologic criteria which perhaps are more important than the histologic appearance of the retina.

I would say that obligatory nocturnality would be one. The rabbit is not such an animal. The lack of Purkinje shift in the electroretinogram would be another criterion, and a maximum of a single spectral sensitivity curve at or around 507 millimicrons would be still another criterion.

An animal which does fulfill these criteria is the Douracouli—the South American night monkey. In some experiments on this animal conducted with Dr. Praglin in our laboratory the Douracouli in a peculiarly perverse manner, showed a double a-wave.

One might postulate a number of reasons for this, possibly two photosensitive rod-type pigments which have different rates of reactivity to light. This cannot be entertained seriously without more evidence, but it is one distinct possibility.

The explanation of the dual effect from alloxan is a difficult one, unless one can demonstrate in the

same animal that the negative response which is presumably obtained from a small-dose effect is followed on additional doses by the low positive response.

This low positive response of approximately 50 microvolts could be distinctly measurable, perhaps, with other equipment; and with absence of noise might show a more detailed picture.

Finally, in the case of the Dithizone experiment, we have had similar experiences in the past. This is presumably based on the insolubility of Dithizone, even in a solvent such as alcohol, and the tendency to form small, highly toxic emboli in the capillary circulation.

DR. DAVID G. COGAN (Boston, Massachusetts): If Dr. Davis told us what the histology was, I missed it. Could he tell us what the sections showed in the eye of that alloxan rabbit with obliteration of the electroretinogram?

DR. ROBERT J. DAVIS (Iowa City, Iowa): I did not mention the histologic changes, Dr. Cogan, because we found no pathologic change in our sections. We attributed this fact to the time factor. We did not feel that our animals had received the drugs over a long enough period of time to bring about a histologic change, although there was a physiologic change.

That is essentially all we have to say about it. We realize there are some unanswered questions.

Our experience with Dithizone was as Dr. Potts pointed out; we were unable to get it into solution. It still remains a mystery to me how the Italian workers who initially performed this experiment were able to do it.

STUDIES ON THE VISUAL TOXICITY OF METHANOL*

VIII. ADDITIONAL OBSERVATIONS ON METHANOL POISONING IN THE PRIMATE TEST OBJECT

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In a previous report from this laboratory,[1] the complete parallelism between symptoms of methyl-alcohol poisoning in the human and in the rhesus monkey was described. In a subsequent report[2] we told how the typical retinal edema, pupillary dilatation, and, in many cases, death occurred in methanol-poisoned monkeys even though the usual metabolic acidosis was completely prevented by administration of base.

There are additional aspects of methanol poisoning in which the primate test object can be used advantageously to help clear up points still in some doubt. One of these areas of confusion deals with the histologic findings in the retina of methanol-poisoned animals. In the first paper, a review was presented of histologic findings in experimental animals without attempting a critical evaluation of the findings presented. Suffice it to say that there are equally vocal adherents of no retinal findings and of marked histologic changes in lower animals poisoned with methanol. Since these animals do not show the typical signs of human poisoning, and since the dose employed is excessive in comparison to the human or the monkey toxic dose, the point is perhaps an academic one. In the case of the single monkey reported by Birch-Hirchfeld[3] and those of Scott, Helz, and McCord,[4] there is no discrimination between findings in the monkeys reported and in the lower animals.

In the cases of human methanol poisoning where eyes were studied histologically, there are similar discrepancies. Roe[5] claims marked changes in the ganglion cells of the 12 patients examined by him; and in the case of one eye fixed rapidly 45 minutes after death, he claims to have controlled post-mortem changes. However, McGregor[6] and Orthner[7] assert with equal positiveness that

* From the Laboratory for Research in Ophthalmology, the Ophthalmology Service, Department of Surgery, and the Department of Pathology, Western Reserve University. Supported in part by grants from the U. S. Public Health Service, National Institute of Neurological Diseases and Blindness, and by the Lions Clubs of Ohio.

no typical changes are found in the retinas of individuals dying of methanol poisoning. Thus, it seems that histologic studies on eyes of valid experimental animals where supply is not a limiting factor and where ophthalmoscopic, clinical, and electric findings would all be available should prove of direct interest.

Another aspect of the methanol problem was emphasized by the finding[2] that despite combating of acidosis, a number of monkeys died in what appeared to be central nervous-system collapse. This once more opens the question of the cause of methanol death and makes advisable studies on the rest of the animal tissues and particularly the central nervous system. This consideration is underlined by the reports of Dozauer[6] and of Orthner[7] of symmetrical lesions in the putamen of patients dying of methyl-alcohol poisoning. Here again, ample supplies of tissues from susceptible animals receiving methanol under controlled conditions would be highly desirable subjects for study.

Finally, in order to bring to the study additional evidence on visual function, the use of electroretinography on the monkeys given methanol and its oxidation products would be highly desirable. It had been shown previously[9] that cats and rabbits receiving minute doses of formaldehyde exhibited marked accentuation of the negative a-wave and obliteration of the positive b-wave of the electroretinogram. For evident reasons it was desirable to investigate the reaction of the primate test object to methanol and its oxidation products.

This report concerns the histologic findings in the eye, brain, and other tissues of monkeys receiving methanol and its degradation products, and electroretinograms on such animals.

Experimental

Methods

Young adult rhesus monkeys were treated with methanol and with base as described previously.[2] In addition several animals were made acidotic by administration of ammonium chloride by stomach tube, and a number of other monkeys were given formate by the same route. Several animals were given formaldehyde by intravenous drip.

At death eyes, whole brain, and samples of lung, heart, spleen, liver, kidney, intestinal tract, and muscle were removed and fixed in formalin. Eyes were imbedded in celloidin, other tissues in paraffin, and all were stained routinely with hematoxylin and eosin. Where indicated, sections were stained with Weigert's myelin sheath stain.

Electroretinograms were recorded as described in Paper IV of this series.[9]

Results

The eyes of six animals which had received 6.0 gm./kg., that is 188 mM of methyl alcohol per kg., were examined histologically. In all of these eyes cystoid degeneration of the external nuclear layer was a constant finding. There is much question whether significance can be attributed to this particular phenomenon. Whereas this may be a histologic manifestation of the observed retinal edema, it may well be a post-mortem artefact. Only a more extended series of eyes with precise control of post-mortem times can answer this point. With one exception there were no observable changes in the ganglion cell layer of the retina despite the reports referred to previously. This exception was monkey No. 3 which alone of all the series lived as long as nine days. This animal had shown severe retinal edema, fixed and dilated pupils, and apparent blindness, but retinal edema had disappeared by the time of death. Histologically, this animal showed patchy demyelinization of the optic nerve and some questionable loss in numbers of ganglion cells in comparison to the other eyes examined. Photomicrographs of the retina and optic nerve of this animal are shown in Figures 1 and 2. These findings may be compared with those of monkey No. 40, which died in 23 hours of methanol poisoning,

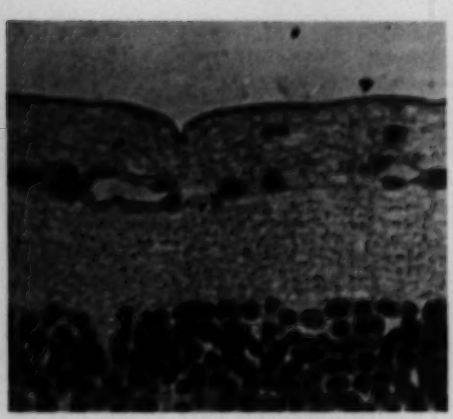

Fig. 1 (Potts, Praglin, et al.) Retina of monkey No. 3. Ganglion cell and inner nuclear layers. (Severe ocular symptoms and nine-day survival.)

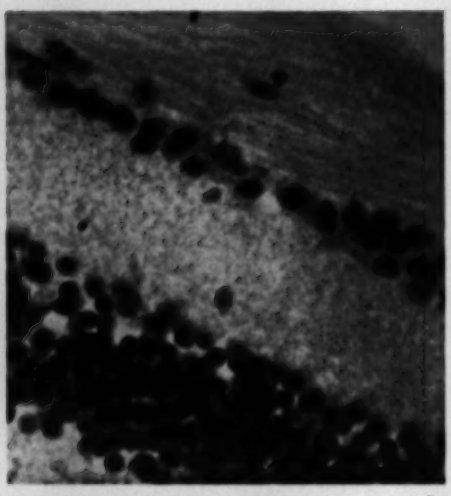

Fig. 3 (Potts, Praglin, et al.). Retina of control monkey. Ganglion cell and inner nuclear layers.

showing marked retinal edema and positive electroretinographic changes.

The whole brain of three monkeys dying of 6.0 gm./kg. methyl alcohol was examined and in each of these marked changes were found. In monkey No. 31, the putamen was grossly involved and to a lesser degree the caudate nucleus (fig. 4). In monkeys No. 34 and No. 36, the damage was again confined to the basal ganglia, but here the caudate nucleus seemed to have sustained the major damage, whereas damage to the putamen was

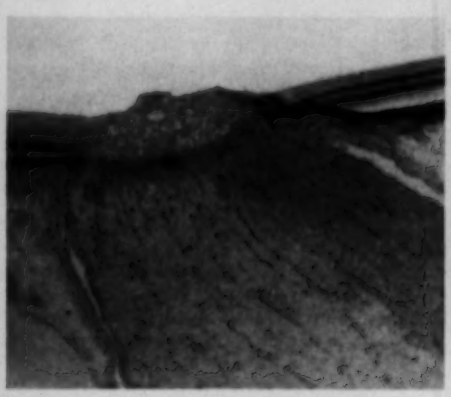

Fig. 2 (Potts, Praglin, et al.). Optic nerve of monkey No. 3, showing demyelinization.

Fig. 4 (Potts, Praglin, et al.). Monkey No. 31, methanol. Gross section through basal ganglia showing necrosis and hemorrhage into putamen.

less significant. Brains of two monkeys dying of intravenous formaldehyde and two monkeys given sodium formate 188 mM/kg. showed no such changes microscopically. The cells in the affected regions show large pericellular spaces. The largest neurons are pale and the nuclei are almost indistinguishable from cytoplasm. The smaller neurons have distorted cytoplasm and eccentric pyknotic dark-staining nuclei and indistinct nucleoli. The intracellular substance of the brain seems coarsened and fibrillar, possibly due to local edema. Comparable sections of the brains of formaldehyde and formate animals showed no such changes. These findings are illustrated in Figures 5 and 6.

Histologic examination of other tissues was performed in five monkeys dying of methyl-alcohol poisoning. In no case were any histologic abnormalities noted in heart, liver, kidney, lung, spleen, skeletal muscle, intestine, or pancreas. In one animal which received ammonium chloride in order to produce acidosis, there were casts found in the collecting tubules of the kidney. In one animal receiving formaldehyde intravenously, a focal chronic interstitial nephritis was observed in the kidney. That these are directly connected with ammonium chloride or formaldehyde administration is certainly not proved although suggested by these findings. In other respects the tissues of formaldehyde animals, formate animals, and ammonium chloride animals were entirely normal.

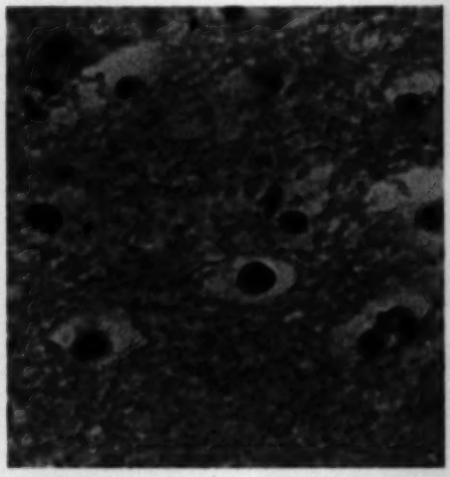

Fig. 5 (Potts, Praglin, et al.) Microscopic section of putamen of monkey No. 31, showing degenerative changes.

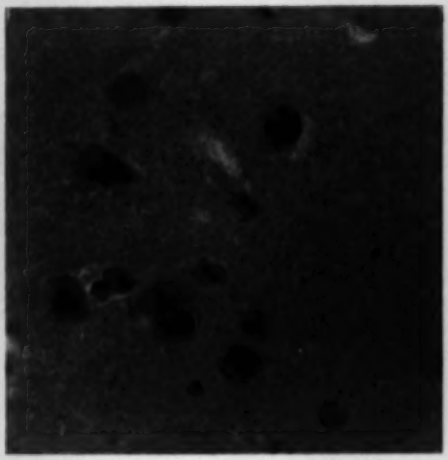

Fig. 6 (Potts, Praglin, et al.). Comparable section of animal killed with formaldehyde. Normal section.

The electroretinogram was measured in seven monkeys dying of 6.0 gm./kg. of methyl alcohol, in three monkeys receiving formaldehyde by intravenous drip, and in three monkeys receiving doses of formate comparable to the methanol doses. In all three cases, the electroretinograms were similar to those elicited in the lower animals, consisting of an accentuated negative a-wave and an absent b-wave. It should be noted that the effect in the methanol animals did not occur until the second day, that is at 20 to 30 hours after administration at a time when most of the methanol had left the body and when the eyes showed visible retinal edema. The formaldehyde and formate effects were immediate, appearing within one to two hours, and obtainable instantaneously by the proper dosage of formaldehyde, as

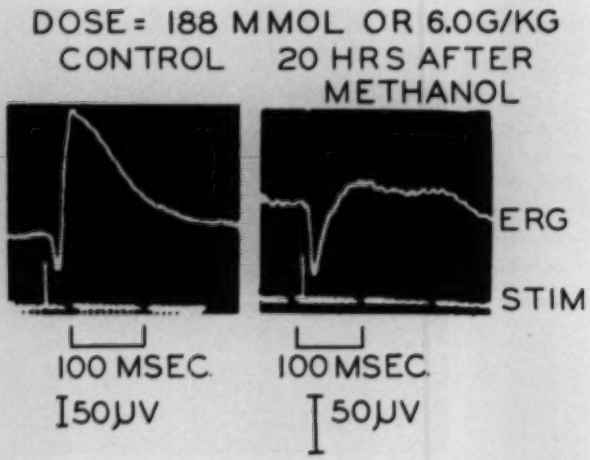

Fig. 7 (Potts, Praglin, et al.). Monkey electroretinogram before and 20 hours after administration of 6.0 gm./kg. methanol.

reported previously. These results are illustrated in Figures 7 and 8.

Discussion

In regard to the histologic eye findings, one fact is outstanding. In our monkeys which showed or had shown severe retinal edema, severe changes in the electroretinogram, pupillary dilatation, and apparent blindness, there were no marked findings. In the one animal which possibly showed changes in the ganglion-cell layer and certainly showed them in the optic nerve survival was an unusual nine days. It is beyond question that one of the late effects in human methanol poisoning is optic atrophy; and

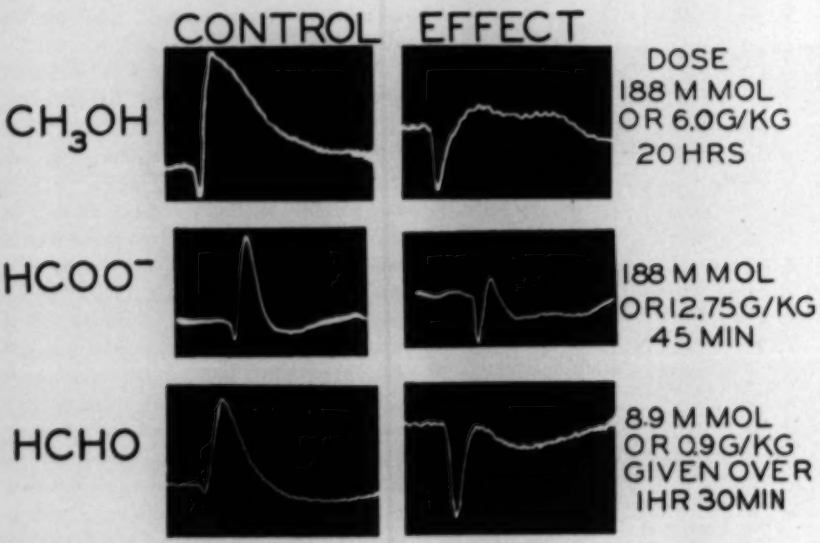

Fig. 8 (Potts, Praglin, et al.). Comparative effects of methanol, formaldehyde, and formate on the monkey electroretinogram.

since the optic nerve represents a collection of axons whose neurons reside in the ganglion-cell layer of the retina, it is reasonable to expect degeneration of the ganglion-cell layer of the retina as a primary finding. However, it is not necessarily reasonable to expect that any such degeneration detected by histologic methods need be visible at the time eye symptoms and ophthalmoscopic findings are at their peak. On the contrary, the lack of change seen in the ganglion cells is ample demonstration of the inadequacy of the histologic method in the face of far-reaching physiologic changes. Cell death does not in itself cause histologically detectable changes since all histologic examinations are done on dead material. It is only when cells are selectively dead long enough to allow degeneration to set in that the histologic method distinguishes these cells from their neighbors and offers a useful contribution.

Such a situation obtained in the case of the brain of methanol-poisoned animals. Here, the time is apparently long enough for damage to the basal ganglia to be evident in stained preparations, and in some cases even in gross specimens. None of the animals in which brain findings were described was allowed to become acidotic. Thus, neither retina change nor brain change can be attributed to the effect of acidosis. One is apparently dealing with yet another specific metabolic effect of methyl-alcohol poisoning. It is important, too, to note that the animals which showed central nervous system lesions, namely the methanol animals, all showed the central nervous-system depression described previously. Several of the animals showed extensor rigidity and tremor, characteristic of basal ganglion lesions. This was particularly true in the case of monkey No. 3.

Here, one has a clue for the first time to the cause of death after acidosis has been combated. Thus, methyl-alcohol poisoning unfolds itself as a complicated phenomenon. First, the narcotic effect of an extremely high dose may cause death of its own accord, and in this respect differs not at all from the other aliphatic alcohols. This is the only phase operative in experimental animals lower than primates. Second, the typical metabolic acidosis, if unrecognized and untreated, may in itself cause death at a later time and after the typical latent period. Finally, even though neither of the first two phenomena may be operative with the dose too low for narcotic death and the acidosis treated with base, still a third cause of death may be the action of a metabolic product on the central nervous system manifested so far principally by the histologic findings in the basal ganglia. One should note once more that the eye effects of methanol poisoning lie in this third phase. Thus, no therapeutic procedure proposed to date can be adequate, since none takes cognizance of this third metabolic poisoning—presumably mediated by a more proximal toxic agent, a metabolic product of methanol itself.

The question presents itself whether the lesions seen histologically in the basal ganglia are an adequate explanation for the late death of the animals. There is little question that such lesions can adequately explain prostration, motor inco-ordination, extensor rigidity, and tremor observed in these monkeys at various times. Whether these of themselves can cause death is another matter and no final answer is possible because of our incomplete knowledge of the physiology of the basal ganglia. It should be noted that the brain stem was examined carefully for histologic changes with negative results. This of course does not exclude other central nervous-system changes—particularly those due to edema and consequent increased intracranial pressure; we can only say no changes were found in our sections.

The electroretinographic findings in the monkeys, as in the nonprimates, show, first, that the electroretinogram may be used as an additional indicator of methyl-alcohol poisoning. This agrees with the report of Karpe (in his discussion of Reference 5) that a similar electroretinogram is found in human methanol poisoning. Retinal edema,

pupillary effects, and electroretinographic changes correlate quite closely. Secondly, when one considers the doses needed to elicit the electroretinographic effect, the formaldehyde is, as before, by far the most potent agent. To date it has not been possible to reproduce the typical central nervous-system findings of methanol poisoning when formaldehyde is given, even by intravenous drip over a period of several hours. This is not too surprising, since here again time for cell death or selective cell death has not been allowed. Further, one still cannot at this stage be certain whether the proximal toxic agent affects the basal ganglia more severely because it is manufactured most readily at that site, or whether the site is most susceptible to its actions. Experiments are now in progress to differentiate these two possible explanations.

Summary

1. The only consistent early change in sections of retinas from methanol-poisoned, bicarbonate-treated monkeys is cyst formation in the external nuclear layer. This is despite marked ophthalmoscopic, pupillary, and electroretinographic changes.

2. The only animal to show possible changes in the ganglion-cell layer of the retina was one which survived nine days. This animal also showed demyelinization of the optic nerve.

3. Methanol-poisoned, bicarbonate-treated monkeys consistently showed edema and nuclear pyknosis in the basal ganglia particularly the putamen and caudate nucleus.

4. When ophthalmoscopic edema and pupillary dilatation set in in these animals, the electroretinogram shows a large a-wave and no b-wave. The same picture may be reproduced with appropriate doses of formaldehyde and formate.

5. The bearing of these findings on our present knowledge of methanol poisoning is discussed.

The authors wish to acknowledge the technical assistance of Mrs. Violet Lima.

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Discussion

Dr. George Wald (Cambridge, Massachusetts): I would like to ask Dr. Potts, though he may have told us, what area of the brain is involved in these lesions?

Dr. Albert M. Potts (Cleveland, Ohio): The area in the basal ganglia is more external than that. The putamen and caudate nucleus are involved. In Orthner's cases, only the putamen was involved; in our cases, there was almost even distribution between the putamen and caudate nucleus.

The whole brain was gone over carefully, and nothing else was found histologically, except in this one area.

The brain was fixed in formaldehyde immediately after removal.

DR. HERMANN M. BURIAN (Iowa City, Iowa): I just want to ask Dr. Potts what light adaptation he used.

DR. POTTS: They were done in dark-adapted animals, ordinarily.

DR. DAVID G. COGAN (Boston, Massachusetts): I would like to ask Dr. Potts if the pupillary reaction would not also have been a satisfactory method of testing their visual function, rather than the electroretinogram, although I realize that, quantitatively, the electroretinogram might have been better.

The other question I would like to ask is: "How does Dr. Potts account for the refractoriness of subprimate animals to methanol blindness?" Maybe he answered that last year.

DR. ALBERT M. POTTS (Cleveland, Ohio): In answer to the first question, the pupillary reaction is a little bit irregular. We have a number of animals which show retinal changes, good retinal edema with a submaximal pupillary response, but with a maximal electroretinogram response. In some cases, we get the so-called cogwheel pupil response rather than fixed dilatation. Last year we talked about this condition, in which the pupil comes up stepwise and comes down stepwise in reaction to darkness or light, respectively.

As far as the explanation for the toxic phenomena is concerned, this is difficult, of course. The obvious hypotheses are available: that local manufacture of the proximal toxic agent—let's say formaldehyde, for the sake of argument—takes place selectively in the retina of the primate or in the liver of the primate, or that the retina of the primate is selectively susceptible to the effect of this toxic agent.

We tend to favor the latter, because we had evidence of good manufacture of formaldehyde in rats when given C_{14} methanol.

DR. WERNER K. NOELL (Buffalo, New York): In our experiments with the previously described poisons, we observe distinct histologic changes, and I am surprised that in the preceding papers, especially in the experiments of Dr. Davis, normal histology was associated with slowly progressing failure of the electroretinogram. It may be that structural changes develop faster in the rabbit than in the monkey or that our conditions are more specific than those in the other rabbit experiments, but damage to the outer limbs is easily overlooked and formalin fixation, for instance, may not reveal early necrosis of the visual cell.

DR. HERMANN M. BURIAN (Iowa City, Iowa): In these experiments with anesthesia, where he got definite changes in the electroretinogram, would you suspect that there was a visible, detectable anatomic effect?

DR. WERNER K. NOELL (Buffalo, New York): Certainly not at all. It depends upon the duration for which the damage is imposed upon the cell. Certainly, one will find nothing histologically if one examines an eye the electroretinogram of which has just been abolished by anesthesia or anoxia.

But the case where the rabbit's electroretinogram has practically disappeared for several days, as shown, makes me wonder why there are no histologic changes. I would expect changes.

DR. ALBERT M. POTTS (in closing): As far as formalin fixation of the brain is concerned (which is the thing we talked about first) the control brains and the experimental brains were fixed in exactly the same way; so this is hardly a fixation artefact.

In connection with the fixation of the eyes, we are doing a set of experiments now, using carotid injection in an animal at the peak of its symptoms. We will have some results from these studies in a while, but do not anticipate marked differences.

RETINAL CHANGES FOLLOWING IONIZING RADIATION*

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AND
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Recent studies[1-4] presented evidence that X radiation of more than 2,000 r is sufficient to exert acute deleterious effects upon the neural structure of the adult mammalian retina. These manifestations of acute radiation retinopathy consisted of immediate and irreversible impairment or abolition of the electroretinogram (ERG). Subsequent pyknosis and autolysis of the rod nuclei developed and led to extensive atrophy and degeneration of the outer retinal layers without the intervention of phagocytes. Principally, the same changes have been observed in the retina of monkeys which had been exposed to other kinds of ionizing radiation as, for instance, Co^{60}-gamma, fission neutrons, thermal neutrons, and a combination of Co^{60}-gamma and fission neutrons.

At this time our presentation will be limited to a discussion of various phases of retinal changes produced by Co^{60}-gamma radiation in two series and by mixed neutron-gamma irradiation in a third series of experiments.

In the first series, 92 monkeys received a whole body gamma radiation of doses varying from 500 to 30,000 r.

In the second series, a fixed dose of 10,000 r Co^{60}-gamma radiation was applied.[5] The radiation was administered either to the head (group I) or to the body (head shielded) (group II), or to the entire body including the head (group III). Each group consisted of 16 animals. In each group two animals were killed by decapitation at fixed intervals varying from two to 96 hours.

In the third series, 25 monkeys were exposed to whole-body mixed neutron-gamma radiation at a ratio of 6:1. The doses employed varied from 2,500 to 30,000 rep and were delivered at high rate from a swimming pool type reactor source.

In series 1 and 2 the dose rate was approximately $1,000 \pm 50$ r/min. The eyes were enucleated as soon as the animals succumbed. They were fixed in Zenker's or Carney's solution and the microscopic sections were stained with hematoxylin and eosin.

Pathologic changes in the retina developed

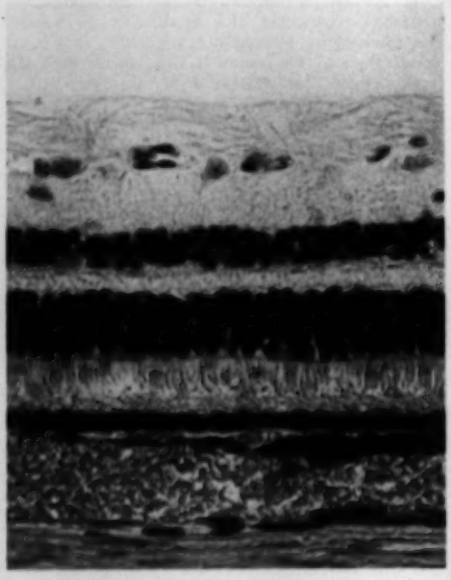

Fig. 1 (Cibis and Brown). Retina of normal monkey (Macaca rhesus). (Zenker, hematoxylin-eosin, ×380.)

* This work has been performed at the USAF School of Aviation Medicine, Randolph Field, Texas, in connection with Project No. 21-3501-0005.

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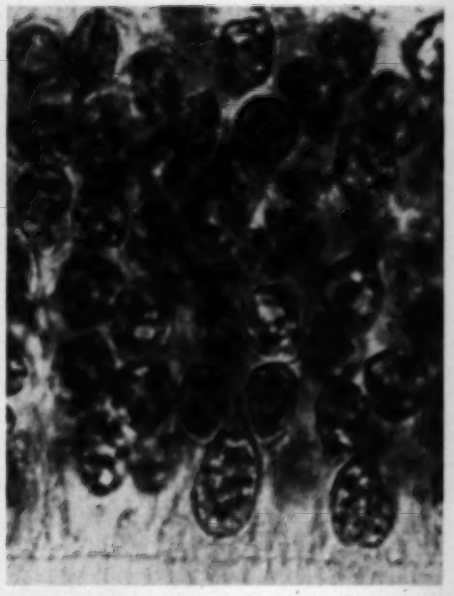

Fig. 2 (Cibis and Brown). Same retina. Nuclei of rods and cones magnified ×1,800. (Zenker, hematoxylin-eosin.)

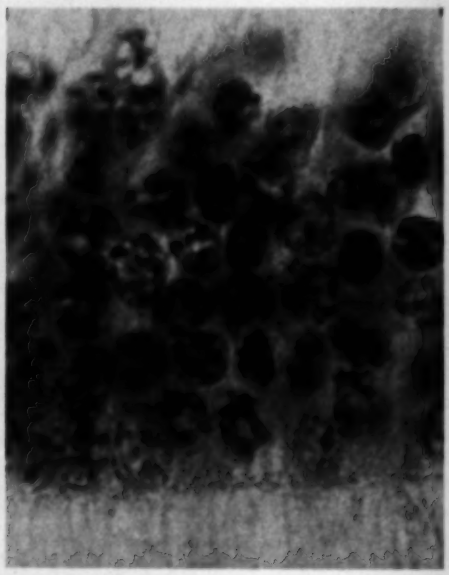

Fig. 3 (Cibis and Brown). Beginning pyknosis of rod nuclei two hours following whole body exposure to 10,000 r Co^{60}-gamma radiation.

only in those cases in which the eyes had been exposed directly to radiation. No changes were found in the group of animals receiving whole body radiation with the head shielded. The appearance of the acute radiation retinopathy in the various experiments was essentially the same as that observed in eyes exposed to equivalent doses of X rays.[4] The main features of microscopic changes observed may be described by the following series of pictures.

Figure 1 depicts the retina and choroid of a control eye. A higher magnification (fig. 2) reveals the normal appearance of the chromatin structure of rod and cone nuclei. As early as 19 minutes following exposure to 30,000 rep mixed neutron-gamma radiation, 25 percent of the rod nuclei exhibited signs of beginning pyknosis. At this dose level, 70 percent of the rod nuclei were pyknotic at 30 minutes, 100 percent at 1.5 hours fol-

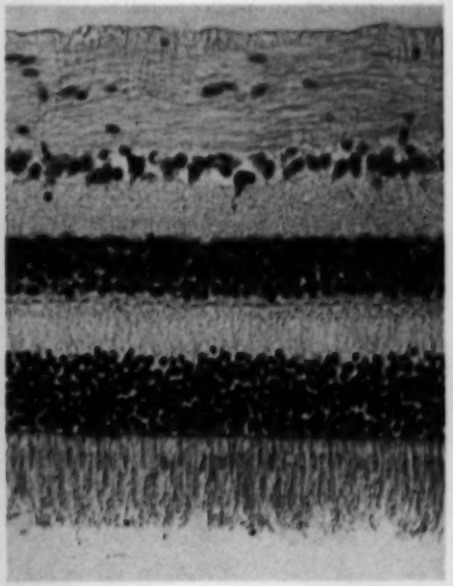

Fig. 4 (Cibis and Brown). Complete pyknosis of about 70 percent of rod nuclei and of about 25 percent of the nuclei of bipolar cells four hours following whole body exposure to 10,000 r Co^{60}-gamma radiation. (Zenker, hematoxylin-eosin, ×260.)

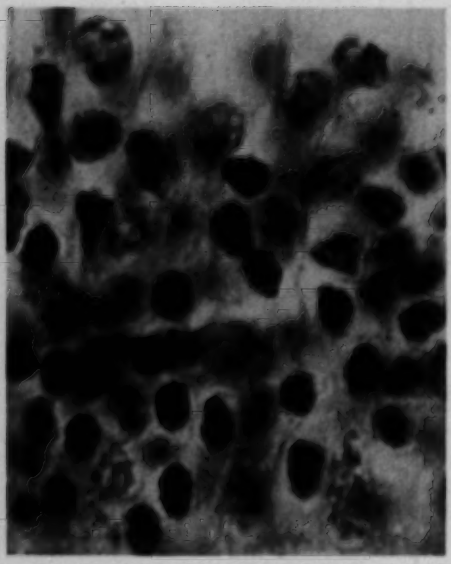

Fig. 5 (Cibis and Brown). Same retina as in Figure 4. Outer nuclear layer magnified ×1,800. Pyknotic nuclei surrounded by edematous cytoplasm. (Zenker, hematoxylin-eosin.)

lowing exposure. When Co^{60}-gamma radiation of equivalent dose was employed, the latent period of pyknotic changes in the rod nuclei was somewhat longer (two to three hours, fig. 3) and more than 70 percent of the rod nuclei were found completely pyknotic four hours following exposure (fig. 4). The nuclei appeared as contracted and misshapen isolated plugs surrounded by a clear zone of edematous cytoplasm (fig. 5). In general, pyknosis seemed to be complete within four hours following exposure.

Edema of the outer retinal layers usually developed concurrently with the karyopyknosis or slightly later. The peak of the edematous changes in the outer retinal layers was reached 48 to 72 hours following exposure. Onset and progression of the retinal edema were, in general, in proportion to the doses employed.

Figure 6 illustrates the retina of a monkey killed 17 hours after exposure to 17,000 rep mixed neutron-gamma radiation. An ad-

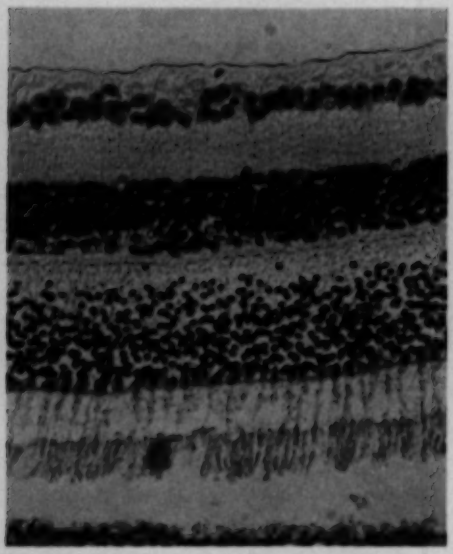

Fig. 6 (Cibis and Brown). Pyknotic and edematous outer nuclear layer of monkey retina 17 hours after whole body exposure to 17,000 rep mixed neutron-gamma radiation. Note edema and advanced disintegration of bacillary layer. Pigment epithelium slightly damaged. (Zenker, hematoxylin-eosin, ×270.)

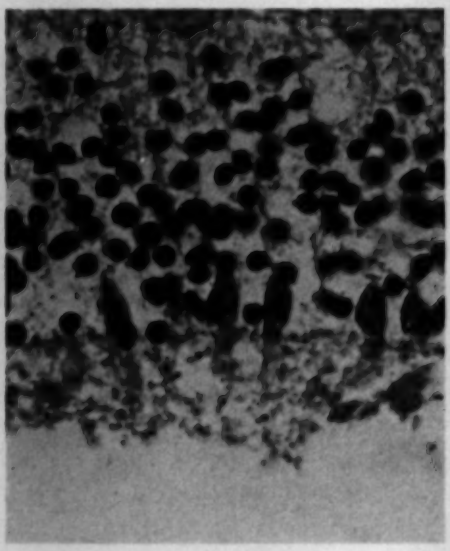

Fig. 7 (Cibis and Brown). Monkey retina 12 hours after exposure to 10,000 *r* gamma radiation. One hundred percent pyknosis of rod nuclei. Normal-appearing cone nuclei with elongated and shrunken inner and outer members. Rod members swollen, disintegrated, or liquefied. (Zenker, hematoxylin-eosin, ×1,800.)

vanced stage of pyknosis and edema of the outer retinal layers was evident. Figure 7 represents the condition of the retina as it appeared 12 hours after exposure to 10,000 r gamma radiation. There was 100 percent pyknosis of the rod nuclei present and the advanced edema of the outer retinal layers was associated with destruction of the bacillary layer including the cone members. The cone nuclei, however, were easily distinguished with apparently normal structure and slightly elongated shape.

Figure 8 illustrates the condition as it appeared 13 days after exposure to 6,000 r gamma radiation to the head. There was advanced atrophy and degeneration of the outer nuclear layers. Only a few pyknotic rod nuclei and a single layer consisting of a cone nuclei remained.

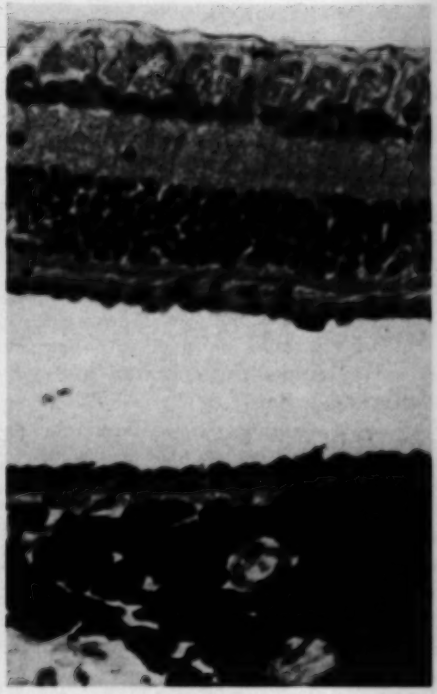

Fig. 8 (Cibis and Brown). Monkey retina 13 days after exposure to 6,000 r Co^{60}-gamma radiation to the head. Advanced degeneration of the outer nuclear layers. Only a few pyknotic nuclei remained. Normal appearance of inner nuclear and ganglion cell layers. (Zenker, hematoxylin-eosin, ×400.)

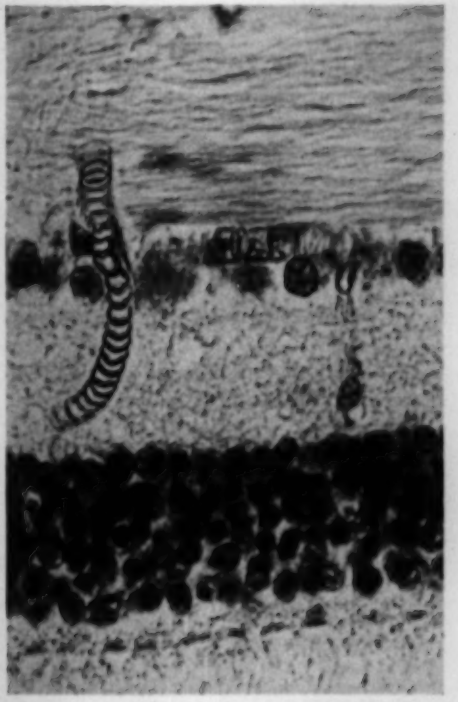

Fig. 9 (Cibis and Brown). Pyknotic bipolar and damaged ganglion cells in monkey retina four hours following exposure to 30,000 rep mixed neutro-gamma radiation. (Zenker, hematoxylin-eosin, ×395.)

Doses of ionizing radiation surpassing 10,000 r directly affected some of the bipolar cells. Doses exceeding 30,000 r or rep usually directly affected all three types of neurons in the retina (fig. 9).

Occasionally vascular changes in the retina and choroid were present and superimposed hemorrhagic and exudative phenomena upon the picture of the previously described acute radiation retinopathy. Nevertheless the edematous changes were usually restricted to the outer retinal layers whenever the radiation had been administered to the eye or head, provided the animal had been killed before circulatory failure and other circumstances of general radiation sickness complicated the picture.

The early changes in radiation retinopathy were characterized by an immediate inhibitory effect on the function of the rod cells and the appearance of histopathologic changes after 19 minutes to four hours. This differed essentially from the late radiation retinopathy resulting from induced aplastic anemia and agranulocytosis or abiotrophic conditions.

This late type of radiation retinopathy was described by Schlaegel[6] and Flick[7] among the casualties of Nagasaki and Hiroshima. The retinal changes in those cases were usually of vascular origin and located in the inner retinal layers rather than in the outer.

SUMMARY

The histologic manifestations of acute radiation retinopathy were studied in a total of 165 monkeys (Macaca rhesus) which were exposed to high intensity gamma or mixed neutron-gamma radiation. The radiation, varying in dosage from 500 to 30,000 r or rep, respectively, was applied either to the head, whole body, or the body with head shielded.

The differentiation of the acute from the late form of radiation retinopathy has been discussed briefly.

USAF School of Aviation Medicine.

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THE ASTROGLIA OF THE HUMAN RETINA*

AND OTHER GLIAL ELEMENTS OF THE RETINA UNDER NORMAL AND PATHOLOGIC CONDITIONS

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Previous neurohistologic investigations of the retina of the human eye were primarily concerned with the ganglion and nerve elements, but largely neglected the elaborate system of glia by which the neurons of the retina are supported as are neurons in general. With the exception of the radial fibers of Müller, not much is known about the glia of the retina under normal and pathologic conditions. Friedenwald[1] stated: "Unfortunately, few extensive studies have been made on the retina with special stains developed in the past 20 or 30 years to demonstrate neuroglia and microglia."

This gap in our knowledge was due to technical difficulties. The conventional histologic procedures or the older metallic impregnations were not suitable for visualization of the glia. However, some of these

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difficulties were recently overcome by Scharenberg[2] who prefected the silver carbonate technique of del Rio Hortega and developed a modification which is simple and reliable and permits impregnation and microphotographic recording of the astroglia of the central nervous system including the retina, under normal and pathologic conditions.

It is the purpose of this contribution to describe the normal astroglia of the human retina and some of its typical changes in pathologic situations of the eye. We shall mention also several other types of glia which have not been previously demonstrated in the human retina.

Histologic description

a. Normal Findings

The order of description follows the layers of the retina proceeding from the inner to the outer layers.

The inner limiting membrane is considered to be an acellular product of the retina (Kolmer[3]). This membrane is intimately connected with the radial fibers of Müller, the brushlike inner ends of which find a firm anchorage in it and penetrate the retina radially (figs. 1 and 4). The inner surface of the inner limiting membrane is partly covered by a discontinuous sheet of scattered flat cells with long interconnected processes, the nature of which is still obscure (Friedenwald[1]). The radially arranged elements of Müller form the skeleton of the retina while more delicate glial elements are woven horizontally into it; both structures together support the neurons and the blood vessels.

In the layer of the optic-nerve fibers which are not myelinated, we were unable to find astroglia. However, there are numerous bipolar glial elements with an elongated nucleus and two long, thin, and straight processes which accompany the nerves, but have apparently no connections with the brushlike inner ends of the elements of Müller (figs. 1, 2, and 3). These bipolar cells are identical with the elements of Remak or lemmocytes (del Rio Hortega[4]). They

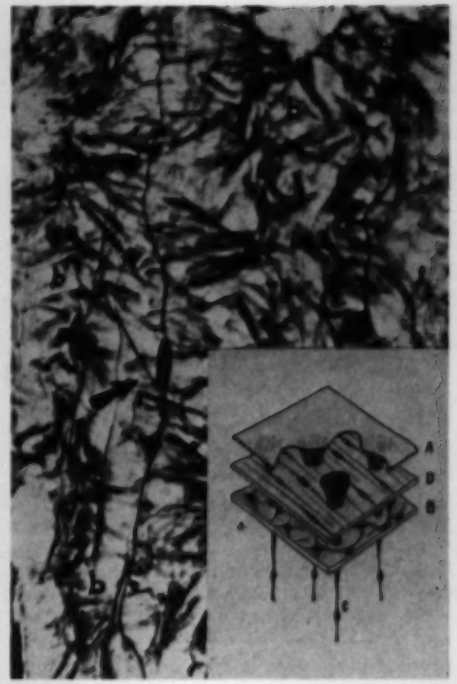

Fig. 1 (Wolter). Lemmocyte (fiber of Remak) of the nerve-fiber layer of human retina (arrow). The nerve fibers are not impregnated. (b) Parts of the brushlike inner ends of the elements of Müller. (Horizontal section, Hortega method, photomicrograph, ×900.) *Inset:* Reconstruction showing the arrangement of the lemmocytes in the nerve-fiber layer (D). (A) Inner limiting membrane. (B) Ganglion-cell layer. (C) Elements of Müller.

are not astrocytes and their nature and significance will be discussed in a subsequent part of this paper. These elements were not found in the area of the optic disque which contains numerous astrocytes with long and branched processes (spider cells).

Besides regular lemmocytes as described above, there are transitional cell forms between lemmocytes and astrocytes at the limit of the nerve fiber layer and ganglion-cell layer. These transitional cells may be found between regular lemmocytes. Usually they possess more than two processes; they are not spindle-shaped but star-shaped (fig. 2-A) and/or form footlike endings on the

Fig. 2 (Wolter). Transitional forms from lemmocytes to astrocytes at the limit between nerve-fiber layer and ganglion-cell layer. (A) Shows two star-shaped lemmocytes (arrows) beside a regular lemmocyte (a). (B) Shows a lemmocyte the process of which ends with a footlike formation on a blood vessel (arrow). (Horizontal sections, Hortega method, photomicrograph, ×900.)

wall of blood vessels (fig. 2-B). These morphologic characteristics are present in astrocytes but not in the typical lemmocytes.

In the ganglion-cell layer there are many well-differentiated astrocytes of a definite type. These are star-shaped elements with a round nucleus and several long and slender processes which maintain an intimate and very characteristic relationship to the blood vessels (fig. 5) and to the neurons (figs. 3 and 4). The astrocytes are arranged horizontally (fig. 4), surround the vessels with a dense network of fibers, and form archlike structures parallel to the vessel wall (fig. 5). Between the vessels of this layer, numerous long processes (glial fibers) of the astrocytes form an interconnected, honeycombed structure of a remarkably regular pattern which surrounds the nerve cells with a protective and supporting network (figs. 3, 4, and 5). This network of the astroglia is definitely placed at a right angle to the coarser structures of the radial fibers of Müller, which as previously stated are arranged radially (fig. 4, also inset fig. 3).

In the inner plexiform layer there are numerous astrocytes which are very similar in type to those of the ganglion cell layer. They are also star-shaped, their processes are numerous, long, and branched, they cross each other repeatedly, and form a dense, horizontally arranged network; but the pattern of this network is irregular and differs greatly from that of the astrocytes of the ganglion cell layer (fig. 6). These astrocytes also show an affinity to the blood vessels on the walls of which their processes form footlike formations (fig. 6).

The inner nuclear layer contains no astrocytes of its own, but numerous processes of the astroglia of the inner plexiform layer enter it with the blood vessels (fig. 7). The absence of astrocytes in the inner nuclear layer is not surprising since most of the space between the neutrons is occupied by the bodies of cells of Müller which are considered to be modified astrocytes (Marchesani[8]) or ependymal cells (Kolmer[3]).

Fig. 3 (Wolter). Dense network of processes of the astrocytes in the ganglion-cell layer of the human retina connecting blood vessels (b) and ganglion cells (a) which are only faintly impregnated. (Horizontal section, Hortega method, photomicrograph, ×600.) *Inset:* Reconstruction showing the arrangement of the astroglia of the ganglion cell layer (B). (A) Inner limiting membrane. (C) Fibers of Müller.

Fig. 5 (Wolter). Architecture of the processes of astrocytes around a blood vessel of the ganglion-cell layer. (c) Faintly impregnated ganglion cells. (Horizontal section, Hortega method, photomicrograph, ×500.)

Fig. 4 (Wolter). Shows the honeycombed network of the astrocytes (a) and the radially arranged brushlike inner ends of the fibers of Müller (b) in a horizontal section of the ganglion-cell layer. (Hortega method, photomicrograph, ×500.)

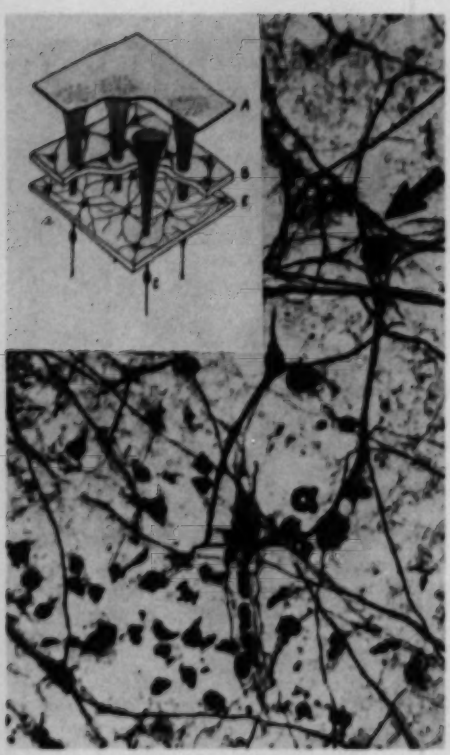

Fig. 6 (Wolter). Astrocyte (arrow) of the inner plexiform layer of human retina with its long and branched processes. One process ends on the wall of a small blood vessel (a). (Horizontal section, Hortega method, photomicrograph, ×900.) *Inset.* Reconstruction of the arrangement of the astroglia in the inner plexiform layer (E). (A) Inner limiting membrane. (B) Ganglion cell layer. (C) Fibers of Müller.

In the outer plexiform layer the astroglia stain-modification of the silver-carbonate technique shows a dense interlacing network of fibers which probably represent processes of astroglia peculiar to this layer; but these fibers could not definitely be traced to the cell bodies (fig. 8). There is no reason to identify these fibers with processes of ganglion cells or of the elements of Müller.

In the outer nuclear layer and in the layer of the rods and cones we could only trace the outer structures of the elements of Müller, which form the outer limiting membrane and the fibrillar baskets. However, no astrocytes were found.

In addition to the perivascular arranged astrocytes as described above, we found in the ganglion-cell layer and in very close proximity to its vessels, an entirely new type of cell of a very complex structure. This cell has an elongated body placed on the vessel wall and numerous powerful processes which surround the vessel with irregularly spaced rings or spirals (fig. 9). These elements seem to be present throughout the other layers of the retina as well. They are similar to the spirocytes of del Rio Hortega[7,8] and Scharenberg.[9]

b. Pathologic findings

The order of description of the pathologic findings on the glia of the human retina follows—so far as possible—the layers of the retina proceeding from the inside to the outside.

The peculiar flat cells on the inner surface of the inner limiting membrane which have been mentioned above and which we tend to consider as glial elements, are extensively increased and hypertrophic in different pathologic situations; for example, occasionally in inflammatory or degenerative involvement of the retina.

The lemmocytes of the nerve-fiber layer seem to be relatively passive in the pathologic occurrences of the retina. Their destruction seems to be their only visible morphologic reaction to retinal damage and usually occurs together with the degeneration of the nerve fibers. We never found hypertrophic or proliferating lemmocytes. As glial scar formation follows the breakdown of the nerve elements of the retina, the lemmocytes are no longer present in the area of the nerve fiber layer but proliferating astrocytes of the ganglion cell layer usually grow into it (fig. 13).

The pathology of the astroglia of the ganglion cell layer and that of the inner plexiform layer are very similar and may therefore be described together. Under se-

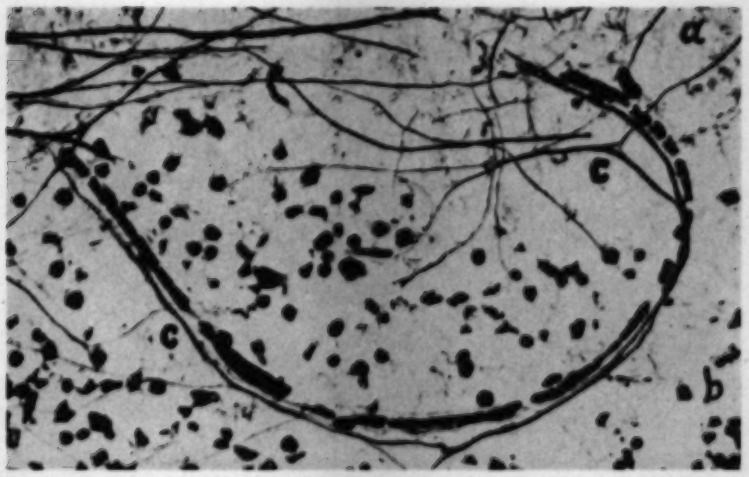

Fig. 7 (Wolter). Processes of astrocytes of the inner plexiform layer (a) which enter the inner nuclear layer (b) with a small blood vessel (c). The vessel wall is not impregnated but the row of erythrocytes is visible. (Hortega method, photomicrograph, ×600.)

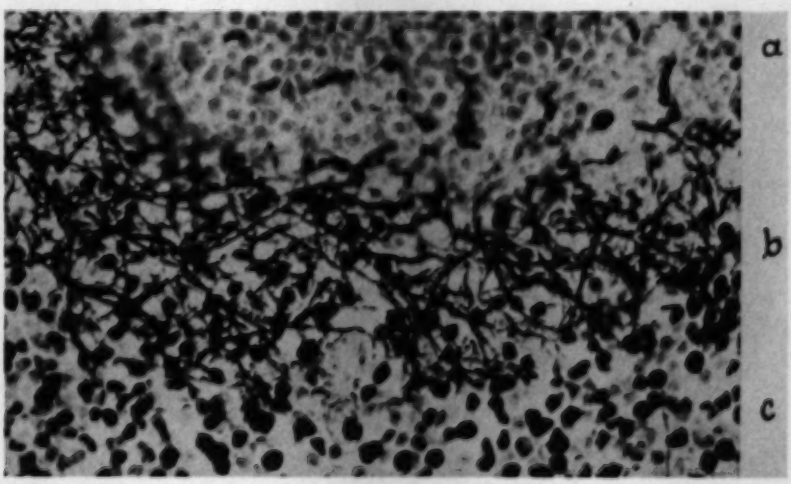

Fig. 8 (Wolter). Dense interlacing network of fibers which probably represent processes of astrocytes peculiar to the outer plexiform layer (b) of human retina. (a) Inner nuclear layer. (c) Outer nuclear layer. (Hortega method, photomicrograph, ×600.)

vere pathologic conditions of the retina with proliferating and hypertrophic astroglia, we have been unable to distinguish the reactive forms of these two only slightly different types of astrocytes.

Within this contribution, the description of the pathology of the astroglia cannot be complete. But we can give examples of the main types of reactions since the changes of the astroglia are unquestionably nonspecific. These main reactions are: Hypertrophy and proliferation, scar-formation, degeneration, and complete destruction.

Figure 10 shows an astrocyte of the retina

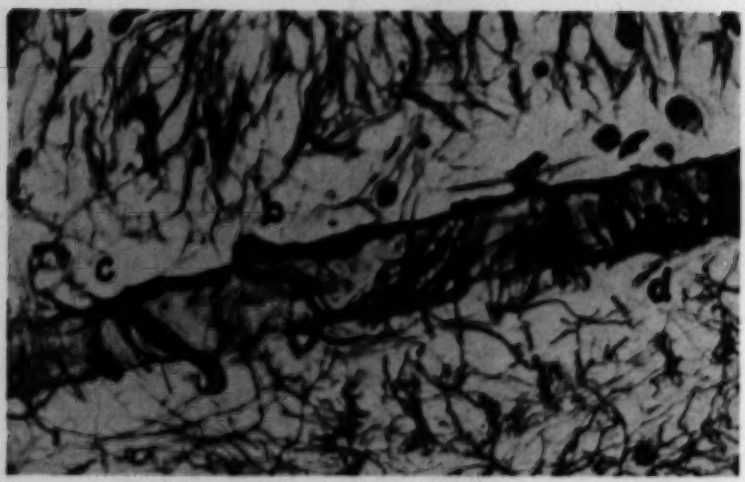

Fig. 9 (Wolter). Glia element of the perivascular type surrounding a blood vessel of the ganglion cell layer of human retina. (a) Spiral-shaped process. (b) Cell body. (c) Handlike structure on the vessel wall which represents another process. (Hortega method, photomicrograph, ×500.)

Fig. 10 (Wolter). Hypertrophic astrocyte in a retina of an eye with absolute glaucoma which shows complete breakdown of the neurons. The hypertrophic cell is about four times as big as a normal astrocyte and sends long, branched processes into the tissue. (Horizontal section, Hortega method, photomicrograph, ×600.)

of an eye in which absolute glaucoma had relatively shortly developed. The neurons of this retina were found to be completely broken down but there was an immense hypertrophy and proliferation of the astroglia. Extremely large astrocytes were noted and their long and branched processes were seen to form a dense, irregular network which filled the space of the broken down neurons (fig. 10). In the area of the virtually completely destroyed pigmented epithelium, the processes of the hypertrophic cells were arranged in a wall-like formation of especially dense fibers (fig. 11). Figures 10 and 11 represent typical astroglia of the retina within the phase of hypertrophy and proliferation which immediately follows the destruction of the neurons in many pathologic situations.

After resorption of the broken-down neurons, the astroglia in many cases survives and forms glial scar. Within this scar we found very irregularly shaped astrocytes. They may form for example the so-called "giant astroyctes" (fig. 12) which represent large cells with a big and irregular nucleus and long and branched processes. However, most of the astrocytes within retinal glial scar shrink to their previous normal

Fig. 11 (Wolter). Processes of another astrocyte (a) of the same retina as in Figure 10. These processes form a dense, wall-like formation against the choroid in the area of the broken-down pigmented epithelium of the retina; (b) retina, (c) choroid. (Hortega method, photomicrograph, ×600.)

Fig. 12 (Wolter). Giant astrocyte with many long processes at the edge of a glial scar in a degenerated retina of a very old man. (Hortega method, photomicrograph, ×500.)

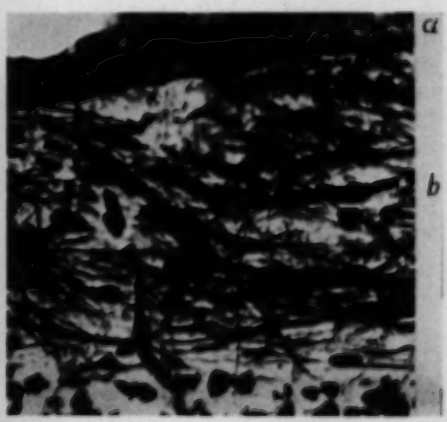

Fig. 13 (Wolter). Glial scar in the area of the inner layers of the retina in a case of old absolute glaucoma. (a) Inner limiting membrane. (b) Dense network of glial fibers (processes of astrocytes) in the area of the nerve-fiber layer, ganglion-cell layer, and inner plexiform layer. The ganglion cells of the ganglion-cell layer have completely disappeared. (Hortega method, photomicrograph, ×400.)

size or even smaller after the hypertrophic phase. Then the cell body is almost invisible and the long processes (glial fibers) mainly represent the scar, forming an irregular and dense network (fig. 13).

In primary or secondary degeneration of the retina there may be hyaline degeneration of astrocytes. Figure 14 shows such an astrocyte, the body of which is replaced by hyaline substance while its processes are still nor-

Fig. 14 (Wolter). Astrocyte of the ganglion-cell layer of the degenerated retina of an old man with hyaline degeneration of its body. The processes are still preserved. (Hortega method, photomicrograph, ×800.)

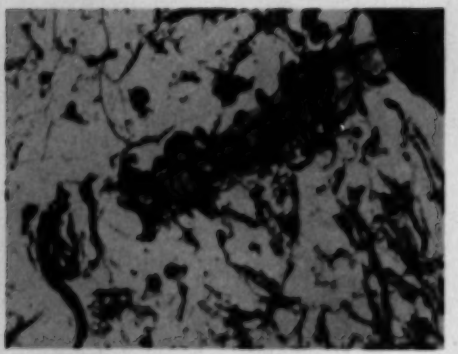

Fig. 16 (Wolter). Perivascular glial element in the degenerated retina of an old man. This cell shows hypertrophy and surrounds the blood vessel irregularly. (Hortega method, photomicrograph, ×500.)

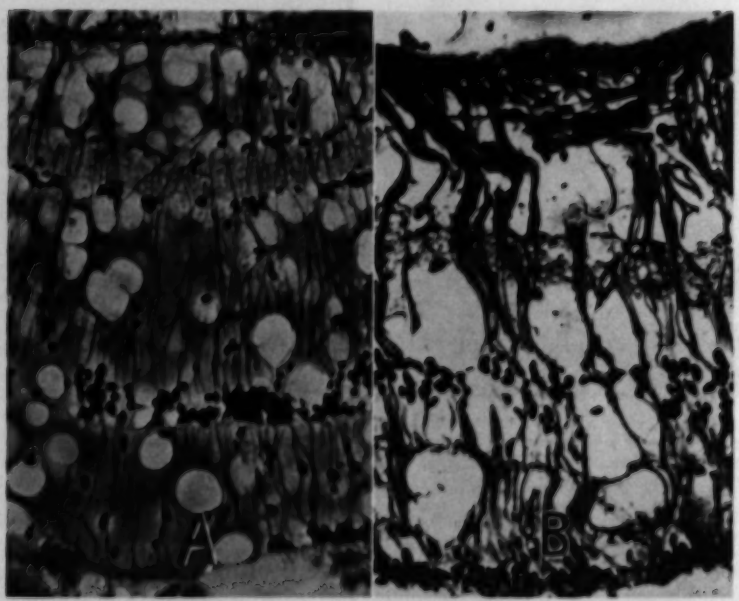

Fig. 15 (Wolter). (A) Beginning—and (B) more advanced cystoid degeneration of the retina of two different cases of retinal detachment. All astrocytes and neurons have disappeared; only the radial fibers of Müller are left. (Hortega method, photomicrograph, ×400.)

mally preserved. In this case the hyaline degeneration occurred in single astrocytes. However, in many cases we also saw spot-like degeneration of complete retinal areas which contained the hyalinized ganglion and glial cells.

In diseases of the retina which cause severe damage, whether this results from an acute or slowly progressive chronic disorder, the astroglia as well as the neurons may be completely destroyed. Figure 15-A and B show sections of the retina of two different eyes with retinal detachment. The general picture is that of a cystoid degeneration of the retina. By our special staining method it is shown that the neurons and all glial elements except the fibers of Müller have disappeared. Generally, the elements of Müller are the most resistant cells of the retina and do not show much reaction in pathologic situations.

We did not see pathologic forms of the astrocytes of the outer plexiform layer, the knowledge of the astroglia of which is still very incomplete.

Fig. 17 (Wolter). "Gittercells" which represent swollen microglia of the retina around a blood vessel in a case of chronic uveitis following corneal injury. These inflated cells contain fat which was demonstrable with "oil red O strain." (Hortega method, photomicrograph, ×500.)

Fig. 18 (Wolter). Rosette from the central area of the retina of a 75-year-old man with typical retinitis pigmentosa. Horizontal section through the scarified outer layers of the retina. (Photomicrograph, ×500.)

The perivascular glia as described above (fig. 9) shows besides those of the astrocytes, the most bizarre reactions in pathologic situations of the retina. Figure 16 shows an example of a hypertrophic perivascular glial cell surrounding a blood vessel in the degenerated retina of an old man. These spiral-shaped structures are very commonly found in different pathologic situations of the retina.

As yet we were unable to demonstrate the normal microglia of the human retina. But in a case of chronic uveitis following an eye injury, we found very numerous "gittercells" (granular compound corpuscles) around the blood vessels of the retina (fig. 17). These cells have a round inflated body and a small nucleus. They contain fat which is demonstrable with "oil red O stain." These gittercells are identical to those seen in pathologic conditions of the brain and are considered to be swollen microglia cells. We found the same type of cell in the optic nerve of a case of neuromyelitis optica.

The significance of primitive glial cells in the pathology of the human retina is still

almost unknown. We usually find what is considered to be primitive glia in the very malignant glioma of the retina (retinoblastoma). However, in a case of retinitis pigmentosa (primary degeneration of the neuroepithelium of retina) in an old man, we also found these undifferentiated cells forming many typical rosettes (fig. 18). Most of these rosettes were present in the area of the macula and around the disque of the optic nerve and were arranged horizontally within the dense glial scar which completely replaced the outer layers of the retina.

Discussion

Five new and morphologically different types of glia were found in the normal human retina: (1) The lemmocytes of the nerve-fiber layer; (2) the astrocytes of the ganglion cell layer; (3) the astrocytes of the inner plexiform layer; (4) the astrocytes of the outer plexiform layer; (5) the perivascular glia.

Detailed knowledge of the glia of the human retina has been thus far restricted to the cells of Müller, including incomplete elements of this type (Cajal,[10] Dogiel,[11] Marchesani,[5] Kolmer,[3] Polyak[6]). These authors used the techniques of Cajal, Ehrlich (methylene blue), the original method of del Rio Hortega, and that of Golgi; but these methods failed to impregnate the lemmocytes, the astrocytes, and the perivascular glia as described here.

As already mentioned there were numerous lemmocytes (cells of Remak) in the nerve-fiber layer. These elements were not demonstrated previously in the retina. According to del Rio Hortega[12] and Scharenberg[13] these cells are intimately connected with the so-called "unmyelinated" nerve fibers of the peripheral nervous system and represent the equivalent of the elements of Schwann which surround the "myelinated" nerves. The presence of the elements of Remak in the nerve-fibre layer of the retina is not surprising since its nerves are not myelinated.

The existence of transitional forms from lemmocytes to astrocytes at the limit between nerve-fiber layer and ganglion-cell layer is surprising. But in spite of this fact we consider lemmocytes and astrocytes as completely different glial elements.

Typical astroglia in the human retina has thus far not been demonstrated. Kolmer[3] stated that there are no astrocytes in the retina proper, although they were found in the papilla of the optic nerve, and was of the opinion that their physiologic tasks are carried out by the cells of Müller. Marchesani[5] expressed the opinion that the retina can be expected to possess an astroglial network but that the original astroglia method of del Rio Hortega failed to impregnate these cells. With the astroglia modification of Scharenberg[2] we were able to confirm this opinion of Marchesani and to visualize various types of astroglia in the retina. Polyak[6] describes radially arranged "fibrous and protoplasmatic astrocytes" in the retina of Chimpanzee and Rhesus macaque (fig. 85, Polyak) but did not mention the dense horizontally arranged network of the astroglia which we found in three layers of the human retina.

The architecture of the astroglia of the retina is most closely adjusted to that of the neurons and the blood vessels and is of definitely different pattern in various layers. The elements of Müller form a coarser skeleton, between the structures of which the delicate network of the astroglia is spread and both must, unquestionably, be regarded as a vital supporting and protecting system of the neurons.

The morphology of the perivascular glia and its relationship to the other elements of the blood vessel wall have not yet been sufficiently studied. How intimate this relationship may be, is indicated by Figure 9; and in pathologic conditions this relationship is even more intimate.

What is known about the pathology of the astroglia and the other glial elements of the human retina is even more limited than their anatomic description. The purpose of this contribution is to describe what we consider to be the main phases of the nonspecific re-

actions of the astroglia. These are: Hypertrophy and proliferation, scar formation, degeneration, and complete destruction. Also we have here described typical pathologic changes of the peculiar flat cells on the inside of the inner limiting membrane and of the perivascular glia. The existence of "gittercells" in a pathologic situation of the human retina is important as an evidence for the presence of microglia in the retina, which could not yet be demonstrated under normal conditions.

We also observed typical rosette formation of retinal elements, which we tend to consider as pathologic glial cells, in the retina of a case of retinitis pigmentosa in an old man. In glioma of the retina the classification of the cells which form the rosettes is still pending; Virchow[14] considered them as primitive glial elements while Flexner[15] and others assumed them to be rudimentary rods and cones or rudimentary neuroepithelium. It is important to note that our case showed the rosettes in the retina of a 75-year-old man and always within the area of hypertrophic and proliferated glia which had completely replaced the neurons of the outer layers of the retina. Besides in glioma of the retina, rosettes have already been described in eyes of normal children (Seefelder, Lindenfeld, Teng and Katzin[16]), in microphthalmos (Jaensch), in coloboma of the choroid (Joy), in cyclopia (Gartner), and in inflammatory processes of the eye (Pusey, Brown, Murakami).

This study must be considered only as a beginning; it is not at all complete since much more has to be done on this subject.

University Hospital.

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Discussion

Dr. Parker Heath (Sullivan Harbor, Maine): Using the silver-carbonate process developed by del Rio Hortega and modified by Scharenberg, Dr. Wolter has supplied light to a dark area. We have been trying to find out something about the glia of the retina for years; and, to my belief, he is the first one who really has given us patterns of normal morphology and a beginning on pathologic reactions.

Previous studies by Polyak show fragmentary

glia in the retina and some relations of Müller's fibers. Dr. Wolter has found at least five different systems of glia. Some perhaps will question whether his glial cells in the inner and outer reticular area are different. However, anybody who has followed his work closely and has seen his material is very much impressed with the authenticity of what he is doing. I, for one, think his contribution is remarkable and very important.

Concerning evaluation of his nonspecific reactions of the glia these will have to await future studies for confirmation. They seem reasonable. Swelling proliferation and ultimate scar formations all fall into channels which can be called nonspecific, and are not unusual with nervous tissue.

We will hope that certain specific reactions will be discovered in due time.

The lemmocytes which he has found for the first time in the nerve-fiber layer are quite consistent with those found elsewhere on nonmedullated nerves.

To me, this has been one of the most interesting and moving presentat'ons. I wish to thank Dr. Wolter very much for the chance to discuss his paper.

Dr. Frederick H. Verhoeff (Boston, Massachusetts): Dr. Wolter spoke of Müller's fibers as modified astrocytes. I have the idea that these two different kinds of cells are independently developed, and that one is not a modification of the other.

Dr. J. Reimer Wolter (in closing): I would like to thank Dr. Heath for his very kindly words and comments.

To Dr. Verhoeff's question I must state that I do not know whether there is a relationship between astrocytes and the fibers of Müller of the human retina. Marchesani considered the fibers of Müller as modified astrocytes while Kolmer assumed them to be modified ependymal cells, but both had no evidence for their opinion. We have seen that in advanced proliferation of retinal tissue the elements of Müller sometimes act and look like hypertrophic astroglia. However, this finding does not prove that astroglia and the fibers of Müller are related. An embryologic study only could clarify the true situation.

THE HYDRATION OF THE CORNEA*

I. The transport of water from the cornea

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When excised pieces of cornea are bathed in aqueous solutions of various osmotic strengths, they swell markedly—often to several times their initial weight. The mechanism by which such swelling is prevented in vivo is little understood. Historically, two major theories were successively advanced to account for the in vivo status. The first held that the normal hydration of the cornea was maintained because the limiting barriers, particularly the epithelium and endothelium, were impermeable to water. The second was based upon the premise that these restricting barriers were impermeable to the major ionic constituents (sodium and chloride) of aqueous and tears, corneal deturgescence being maintained by an osmotic differential. Aside from certain theoretic objections to such hypotheses, the clinching arguments against them were delivered by demonstrations that the endothelium and epithelium pass both water[1] and sodium[2-5] and presumably the common anions, as well.

In the face of this evidence, the concept has developed recently that the cornea is capable in vivo of actively excreting a major ion (such as sodium or some anion) or, perhaps, water. By inference, this excretion would require work, so would depend upon the metabolism of the cornea. It is the purpose of this communication to report experiments undertaken to determine whether an active process controls the normal ion and water balance of the cornea and whether an ion or water (or both) are actively moved.

To achieve this end we have applied a technique similar to that found useful in the study of the cation and water balance of

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the lens.[6-10] Essentially, it consists of reducing for a time the metabolism of a tissue by refrigeration at 0°C., then restoring the metabolic rate to approximately normal by raising the temperature to 37°C. During refrigeration the ionic composition approaches that of a true equilibrium; subsequent incubation at 37°C. affords an opportunity for metabolic processes to effect a return toward normal. In these studies, the cation and water contents are measured before and after refrigeration and after an additional period of incubation at 37°C.

Methods

Rabbit eyes were used throughout. They were generally obtained from a slaughter house, the eyes being enucleated immediately after decapitation of the animals and transported at once to the laboratory. In certain instances, eyes from animals freshly killed in the laboratory were used for comparison. Each eye was placed in a tube, cornea up, on some folded gauze saturated with a modified Tyrode's solution.[7] The tube was flushed with a mixture of 95-percent oxygen —five-percent carbon dioxide, tightly stoppered and refrigerated in an ice bath for the appropriate period. One cornea of a pair of eyes was analyzed upon removal from the ice bath; the other eye subsequently was incubated at 37°C. for an additional period, then analyzed. This basic procedure was varied frequently, as indicated in the text, to permit an evaluation of specific factors.

For analysis, the eye was hemisected near the equator, the lens and anterior uveal tissue gently removed, and the endothelial surface of the cornea blotted lightly to prevent contamination with aqueous fluid.* A central section (nine mm. in diameter) of the cornea was then taken, using a surgical trephine. The corneal section was weighed immediately, dried at 105°-110°C. to constant weight (48 hours), dry-ashed (with the aid of a small amount of sulphuric acid) at 500°C., and the sodium and potassium contents determined, using a Baird Associates flame photometer with an internal standard. Results are expressed as cation or water *content* (mEq. or moles per 100 gm. of dry cornea) or cation *concentration* (mEq. per 1,000 gm. of cornea water). Glucose was determined by the method of Somogyi.[11]

* It was to minimize such contamination of the cut corneal surface with aqueous fluid that we initially avoided trephining the corneal section from the anterior surface of the intact eye. However, experience proved that when the sections were taken rapidly and with a sharp instrument, the results obtained from either approach were essentially the same.

Cation concentration and water content of the normal rabbit cornea

The normal cation concentrations and water content of the cornea were determined to be essentially the same in rabbit eyes secured at the slaughter house and eyes from rabbits freshly killed in the laboratory. The combined results are given in Figure 1. Values of these constituents in aqueous humor and lenses of rabbit eyes are presented for comparison. (They were not determined by analysis of tissues and fluids from the same eyes.) It is apparent that the concentration of total base (sodium plus potassium) is considerably higher in the cornea than in the aqueous. The analyses here reported, of course, were of the entire cornea, including both cellular and noncellular elements.

Maintenance of cation content and hydration of the cornea during incubation at 37°C.

Before valid temperature reversal studies could be made, means had to be devised for maintaining in vitro the cation and water contents of the cornea as they exist in vivo. Several experimental situations were studied. When enucleated eyes were totally immersed in a modified Tyrode's solution and incubated at 37°C., the sodium and water contents of their corneas increased. Similar results were obtained when the anterior por-

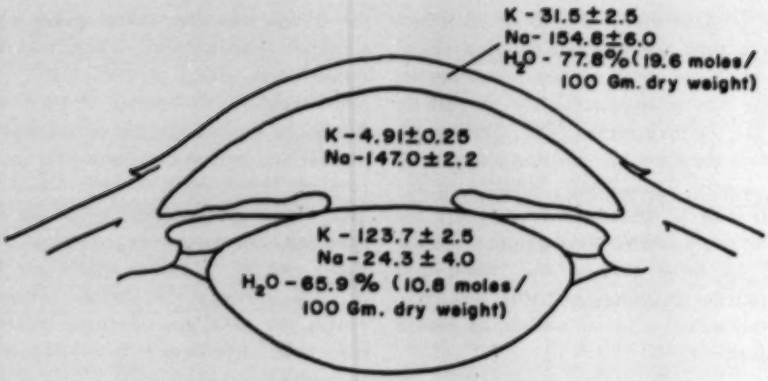

Fig. 1 (Harris and Nordquist). Normal cation concentration (mEq. per 1,000 gm. of water) and water content of the cornea, lens, and aqueous of rabbit eyes with indicated standard deviation. The figures for the cornea represent the average of 36 analyses, those for the aqueous, eight analyses, and for the lens, 62 analyses of lenses weighing between 0.240 and 0.400 gm.

tions of hemisected eyes (devoid of lens and vitreous but with an intact ciliary body and iris) were incubated in this solution (fig. 2). It is plausible that the uptake of water and cation under these circumstances occurs through the limbus; a possible exchange across the epithelium must also be considered.

However, when enucleated eyes were incubated at 37°C. in moist chambers as already discussed, the cation and water contents of the corneas remained reasonably constant for a period of 12 hours (fig. 3). Distillation of water to or from the corneas was insufficient to alter their composition even when the vapor pressure of the atmosphere was varied from that of pure water to that approximating the aqueous humor. (The vapor pressure of the cornea is probably lower than that of the solutions employed. Distillation to the cornea, rather than evaporation from it, thus would be anticipated.)

Effect of refrigeration at 0°C. on corneal hydration and cation content

When rabbit eyes were refrigerated at 0°C. in moist chambers, the water, sodium

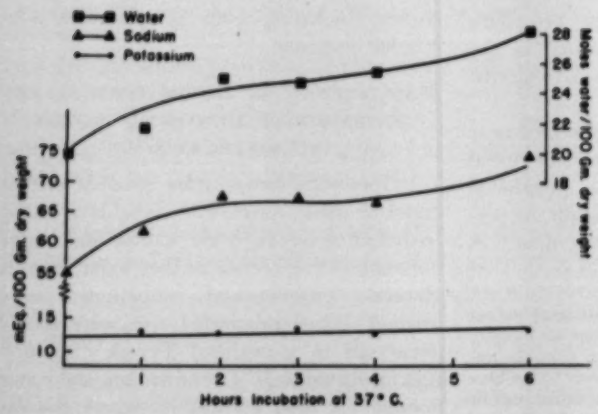

Fig. 2 (Harris and Nordquist). Changes in the cation and water contents of the rabbit cornea during incubation at 37°C. of the anterior half of the eye immersed in a modified Tyrode's solution. The ordinate on the left represents cation content; that on the right, water content. Each point represents an average of two to five analyses.

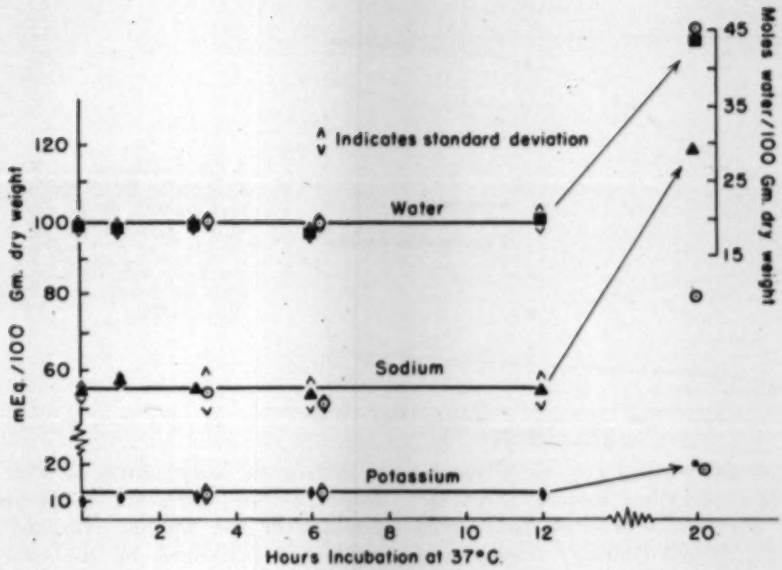

Fig. 3 (Harris and Nordquist). Changes in the cation and water contents of the rabbit cornea during incubation in a moist chamber at 37°C. of the intact, enucleated eye. The open circles represent the data obtained when a vapor pressure of distilled water was employed. In all other instances a modified Tyrode's solution was used. The ordinate on the left represents cation content; that on the right, water content. Each point is an average of at least six determinations.

and potassium *contents* of the corneas increased markedly (figs. 4 and 5).* However, the net exchange was such that a decrease in *concentration* of the sodium and potassium

*Although not plotted here, analyses were also made after only 12 hours' refrigeration to provide direct comparison with those experiments in which the eyes were incubated at 37°C. After this period in the cold, the water content had risen to 27.1 moles per 100 gm. of dry cornea and the sodium and potassium respectively to 72.0 and 13.1 mEq. per 1,000 gm. of cornea water.

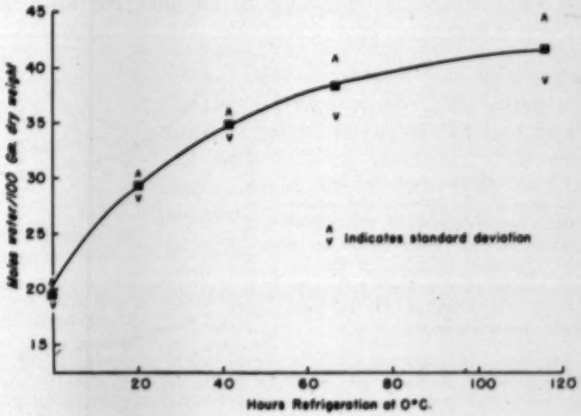

Fig. 4 (Harris and Nordquist). Changes in hydration of the rabbit cornea during refrigeration in a moist chamber at 0°C. of the intact, enucleated eye. Each point represents the average of at least seven determinations.

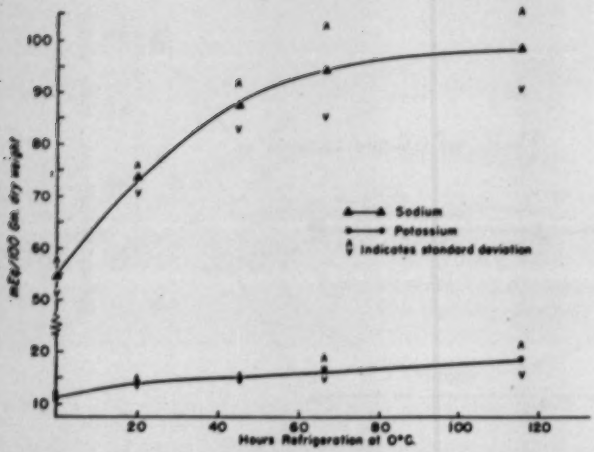

Fig. 5 (Harris and Nordquist). Changes in the sodium and potassium contents of the rabbit cornea during refrigeration in a moist chamber at 0°C. of the intact, enucleated eye. Each point represents the average of at least seven determinations.

of the corneas resulted (fig. 6). (That the curves in Figure 6 are biphasic is not surprising. The concentration of these cations in the aqueous varied during refrigeration of the intact enucleated eye, sodium being lost from the aqueous to cellular structures, particularly the lens, and potassium being gained from them. This probably accounts for the rise in concentration of potassium in the corneas during the later phase of refrigeration.)

The question arises whether this movement of water and cations represents a migration of bulk aqueous or a preferential movement of water. In other words, does the water follow the migration of an ion or is it moved independently by some other force? During refrigeration, the osmotic pressure of the aqueous remained quite constant as measured by the total base (sodium plus potassium) concentration (fig. 7). It can be calculated that the total base of the cornea was diluted more than would have been the case if the dilution had resulted only from the movement of aqueous humor. Such calculation reveals that after 20 hours of refrigeration the total base (sodium plus potassium) of the cornea would be 175 mEq. per 1,000 gm. of water if the increase in hydration represented an uptake of aqueous; actually, a value of only 165 mEq. per 1,000 gm. was obtained. In

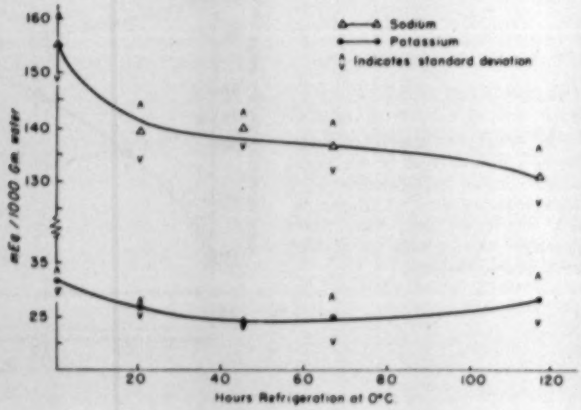

Fig. 6 (Harris and Nordquist). Changes in the sodium and potassium concentrations of the rabbit cornea during refrigeration in a moist chamber at 0°C. of the intact, enucleated eye. Each point represents the average of at least seven determinations.

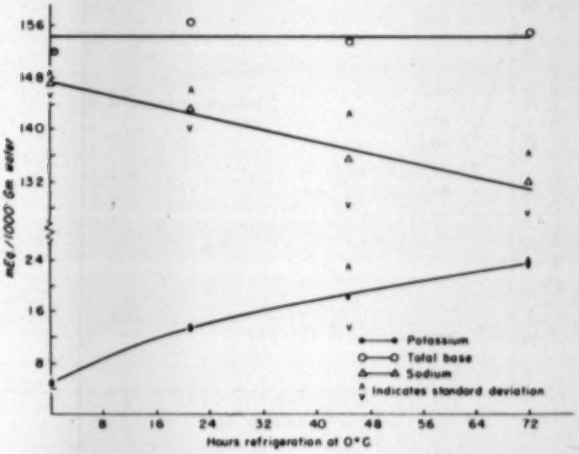

Fig. 7 (Harris and Nordquist). Changes in the concentration of sodium, potassium, and total base of the aqueous humor during refrigeration in a moist chamber at 0°C. of the intact, enucleated rabbit eye. Each point represents the average of at least seven determinations.

other words, during the first 20 hours, 20 percent of the hydration can be construed as due to the movement of water and 80 percent to the movement of aqueous humor. This independent migration of water into the cornea occurred mainly within the first 20 hours; thereafter, the swelling was due largely to the movement of fluid having the total base composition of aqueous.

These changes in hydration and cation concentration in the cornea are quite the opposite of those which occur when a cellular structure, such as the erythrocyte or the lens, is refrigerated at 0°C. In these instances, a decrease in potassium and an increase in sodium content occur as the cations approach equilibrium with the extracellular environment. However, the net exchange is such that in the lens, for example, an increase in *concentration* of total base (sodium plus potassium) occurs (fig. 8). Likewise, an increase in total cation *content* (determined on the basis of dry lens weight) is noted. In the lens, then, in contrast with the cornea, hydration during refrigeration can be accounted for solely by the movement of cation (and anion, as well).

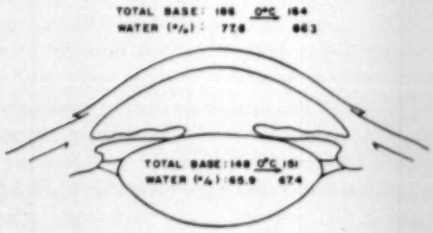

Fig. 8 (Harris and Nordquist). Comparison of changes in the total base concentration and water content of the rabbit lens and cornea during refrigeration at 0°C. for 40 to 45 hours. The data on the cornea were obtained from 17 analyses of corneal sections of intact, enucleated eyes refrigerated in a moist chamber at 0°C. Those of the lens were obtained from 72 analyses of isolated lenses refrigerated at 0°C. in three ml. of modified Tyrode's solution.

Effect of refrigeration and subsequent incubation at 37°C.

When the refrigerated eyes were subsequently incubated at 37°C. the changes in corneal composition which occurred in the cold were reversed, generally.* The water content decreased markedly (fig. 9). As previously mentioned, experiments in which the eyes were incubated at 37°C. without prior refrigeration demonstrated that this dehydration of the cornea would not be due

* Incubation at 37°C. was usually terminated at the end of six hours, since by then the glucose concentration of the acqueous had fallen to substantially zero.

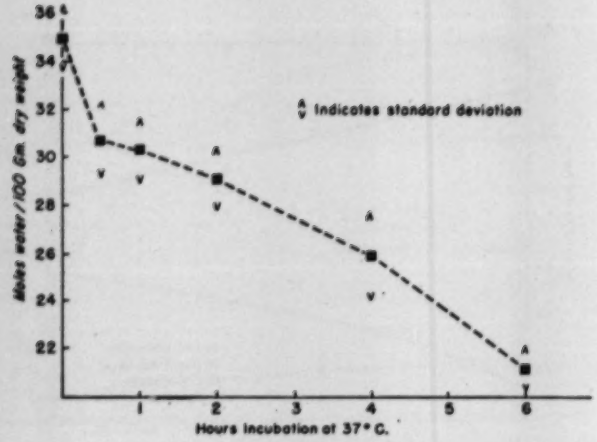

Fig. 9 (Harris and Nordquist). Change in the water content of the rabbit cornea during incubation in a moist chamber at 37°C. subsequent to 45 hours' refrigeration at 0°C. of the intact, enucleated eye. Each point represents the average of at least eight determinations.

to simple evaporation. The sodium content also decreased, while the potassium remained quite constant (fig. 10). However, the sodium *concentration* rose rapidly early in the period of incubation at 37°C. following refrigeration for 45 hours, then tended to stabilize as the normal sodium level was approached (fig. 11). Thereafter, corneal water and corneal sodium decreased at the same rate, resulting in a fairly constant sodium concentration there. (The sodium concentration in the aqueous was rising simultaneously.) These data suggest that the initial dehydration of the cornea was due solely to water movement. This was best illustrated by the changes during incubation following only 20 hours' refrigeration (fig. 12). Here a marked decrease in water content was demonstrated clearly in the early period of incubation while the cation content remained relatively constant.

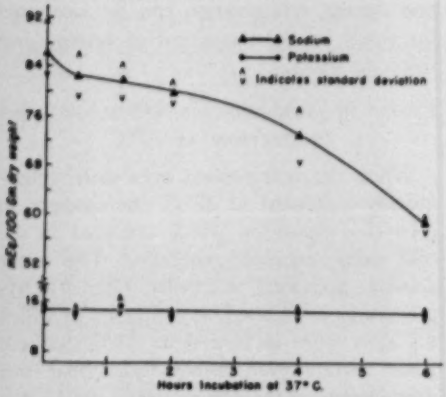

Fig. 10 (Harris and Nordquist). Changes in the sodium and potassium contents of the rabbit cornea during incubation in a moist chamber at 37°C. subsequent to 45 hours' refrigeration at 0°C. of the intact, enucleated eye. Each point represents the average of at least seven determinations.

Effect of anaerobic conditions

More direct evidence that this dehydration depended upon metabolic processes was obtained by depriving the eyes of oxygen. Under anaerobic conditions (95-percent nitrogen—five-percent carbon dioxide), the previously refrigerated corneas were unable to excrete water or sodium during incubation at 37°C. (fig. 13). Similarly, without atmospheric oxygen the corneas of eyes not previously refrigerated were incapable of maintaining their normal cation and water relationships during incubation at 37°C. In 12 hours of such incubation, corneal water rose from 20 to 45 moles per 100 gm. dry weight, sodium increased from 55 to 119, and potassium from 11 to 19 mEq. per 100 gm. dry weight. These latter experiments, like the in vivo studies of Smelser and Ozanics,[12] support the view that at least part of the

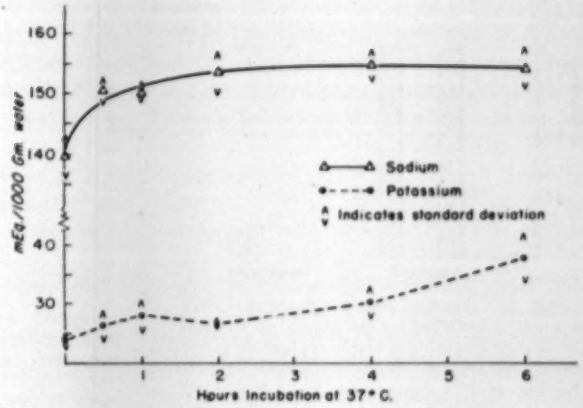

Fig. 11 (Harris and Nordquist). Changes in the sodium and potassium concentrations of the rabbit cornea during incubation in a moist chamber at 37°C. subsequent to 45 hours' refrigeration at 0°C. of the intact, enucleated eye. Each point represents the average of at least seven determinations.

oxygen required for corneal metabolism may be derived from the atmosphere.[13]

Relationship of sodium to water movement

By a variety of procedures, one can alter the ability of the cornea to recover its normal cation and water relationships following refrigeration. Two such procedures are reported here to demonstrate that the movement of sodium and water are closely associated. In the first, 31°C. (the temperature employed in similar experiments by Davson[14]) was used instead of 37°C. as the incubation temperature for the recovery phase of the experiments. The excretion of both water and sodium was diminished at the lower temperature (fig. 14). The fact that the water content more nearly approaches normal than does the sodium content might be predicted from the studies of kinetics mentioned above. Such data suggests that closing the eyelids may promote dehydration of the cornea by raising its temperature.

In another series of experiments, approximately two thirds of the corneal epithelium was removed mechanically by scraping the surface with a sharp blade. These corneas gained water and sodium to the usual extent during refrigeration. However, during subsequent incubation return toward nor-

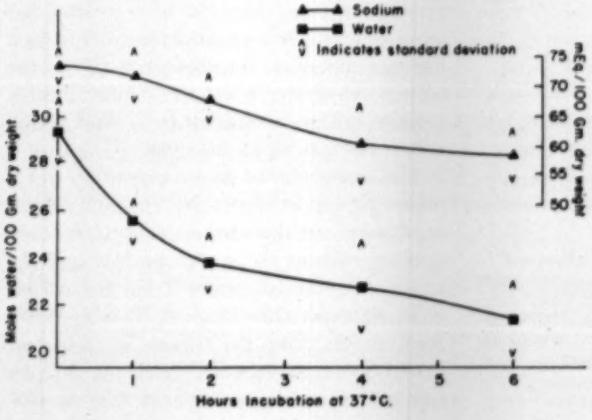

Fig. 12 (Harris and Nordquist). Changes in the sodium and water content of the rabbit cornea during incubation in a moist chamber at 37°C. subsequent to 20 hours' refrigeration at 0°C. of the intact, enucleated eye. The ordinate on the left represents the water content; that on the right, the sodium content. Each point represents the average of at least seven determinations.

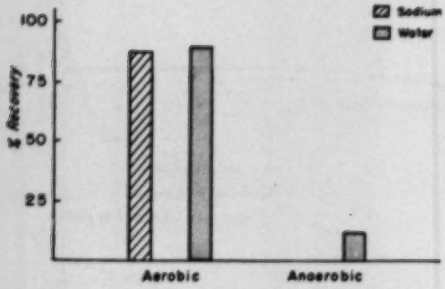

Fig. 13 (Harris and Nordquist). Effect of anaerobic conditions on the degree of recovery of the normal sodium and water content of the rabbit cornea during six hours' incubation in a moist chamber at 37°C. subsequent to 45 hours' refrigeration at 0°C. of the intact, enucleated eye. The percent recovery is calculated using the formula $\text{percent recovery} = \frac{C_r - C_i}{C_r - C_n} \times 100$ where C_n is the content of the normal cornea and C_r and C_i represent respectively the content of the cornea following refrigeration and following refrigeration plus incubation at 37°C. Each bar represents the average result of at least seven experiments.

mal of both water and sodium was reduced, with sodium again lagging behind (fig. 15).

Comment

The results leave little doubt that the corneal hydration is normally maintained by an active metabolic process. This process does not appear to result directly from some

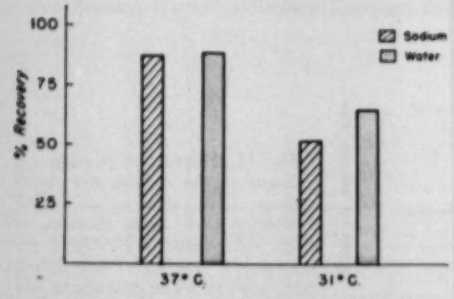

Fig. 14 (Harris and Nordquist). Comparison of degree of recovery of the normal sodium and water contents of the rabbit cornea during six hours' incubation in a moist chamber at 31°C. and 37°C. subsequent to 45 hours' refrigeration at 0°C. of the intact, enucleated eye. Each bar represents the average result of at least eight experiments. For method of calculation see Figure 13.

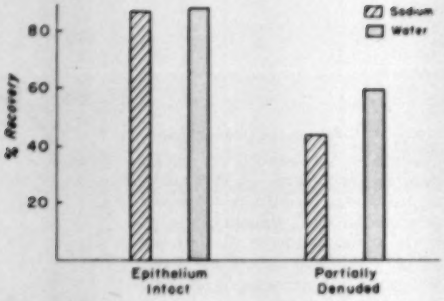

Fig. 15 (Harris and Nordquist). Effect of partial de-epithelization of the rabbit cornea on the degree of recovery of its normal sodium and water contents during six hours' incubation in a moist chamber at 37°C. subsequent to 45 hours' refrigeration at 0°C. of the intact, enucleated eye. Each bar represents the average result of at least seven experiments. For method of calculation see Figure 13.

action on the collagen fibers, since hydration occurs at either 0°C. or 37°C. when an excised cornea or the entire eye is immersed in solution. Moreover, when oxygen was excluded the active excretion of fluid from the cornea was inhibited. Thus, both a barrier (or barriers) and an energy source are essential to the maintenance of the normal corneal hydration.

The substance which is transferred actively or pumped from the cornea appears to be water rather than one of the major cations (or anions). The fact that the cations of the cornea are diluted during refrigeration more than can be accounted for by a bulk movement of aqueous and that during subsequent incubation at 37°C. the cornea dehydrates while the sodium content remains relatively constant for a short period is best interpreted in this light.

The changes in corneal composition which occur during refrigeration and subsequent incubation can thus be analyzed as follows. Cooling reduces the metabolic rate and the active excretion of water from the cornea is at least partially blocked. Water, therefore, moves into the cornea in response, probably, to an osmotic gradient. Sodium and potassium also enter as a true equilib-

rium is approached and the cornea continues to swell because of the higher osmotic pressure of the protein-containing stroma. The same sequence must occur when oxygen is excluded. When the metabolic rate is returned toward normal by increasing the temperature, water is excreted from the cornea and the cation concentration rises. Movement of sodium from the cornea subsequently occurs by diffusion along a concentration gradient. (During six hours' incubation of intact eyes at 37°C., following refrigeration for 45 hours, the sodium concentration of the aqueous rises from 139 to 146 mEq. per 1,000 gm. of water. The exact concentration of sodium in extracellular corneal water is not known with certainty. During refrigeration, sodium is gained from the aqueous and lost to the corneal cells. During subsequent incubation at 37°C., the sodium concentration in the extracellular phase increases because of the movement of the ion from the corneal cells and the migration of water from the cornea. A concentration gradient favoring the diffusion of sodium from the cornea to the aqueous is thus not unreasonably postulated.)

The fact that the potassium content of the cornea remains relatively constant during incubation at 37°C. does not mitigate against this view. In six hours' incubation following 45 hours' refrigeration, the potassium concentration of the aqueous falls from 19 to only 12 mEq. per 1,000 gm. of water. Thus at the end of the experimental period concentration of potassium in both the aqueous and cornea is in excess of normal and it can be calculated reasonably that a diffusion equilibrium exists.

In vivo, as a result of the excretion of water, a steady state must obtain, in which the corneal stroma is hyperosmotic with respect to aqueous. This view was advanced by Davson[15,16] from analyses of the cornea, although not accepted by Maurice.[2] Our cation values of the normal rabbit cornea agree with those obtained by Davson for the ox cornea, with the exception that we found a slightly higher sodium concentration (155 as against 149 mEq. per 1,000 gm. of cornea water). The concentration of total base (sodium and potassium) of the extracellular fluid cannot be calculated with certainty because of lack of precise knowledge of the cation distribution in the cellular elements. However, it is not unreasonable to assume that concentration of total base of the stroma is at least as high as that of the cellular elements. Accepting then an extracellular total base of 186 mEq. per 1,000 gm. of water, and assuming that the cation : anion (chloride plus bicarbonate) ratio which Davson found for the whole cornea maintains in the stroma, the total osmotic strength contributed by these major cations and anions amounts to 327 mEq. per 1,000 gm. of water, a figure in excess of the total osmotic strength of the aqueous humor.

Lastly, it seems likely (although not proved) that the majority of exchanges which we have demonstrated occur across the endothelial surface. However, the epithelium also plays an essential role in the maintenance of the corneal hydration as demonstrated here and by the work of Maurice and Giardini.[17] The role of the epithelium will be considered more fully in a future publication.

Summary

1. The effect of a reversible alteration of the metabolic rate of the cornea on its water and cation composition has been studied.

2. During refrigeration of the intact, enucleated eye in a moist chamber at 0°C., the cornea becomes hydrated due, initially, to a preferential movement of water and, subsequently, to a movement of fluid having approximately the composition of aqueous humor.

3. During subsequent incubation at 37°C. these changes are reversed, water initially and then sodium plus water moving from the cornea, the potassium content remaining elevated.

4. This reversal is abolished in the absence

of oxygen and reduced when the epithelium is removed partially, or an incubation temperature of 31°C. employed.

5. It is concluded that the normal hydration of the cornea results from an active transport of water from the cornea and that such migration of cations to or from the cornea as normally occurs results from simple diffusion exchange or movement along a concentration gradient.

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Discussion

Dr. Bernard Schwartz (Iowa City, Iowa): Two years ago, at the association meeting, we discussed the matter of the role of metabolism in hydration of the lens and cornea. Although we did not measure the sodium or potassium components at the time, we did measure the weight gain under decreased temperature and anoxic conditions, and discussed this change in terms of active water transport.

It appears that Dr. Harris' experiments confirm and substantiate experiments which we have done and those of Dr. Davson recently in England. I am glad to see that the thinking is in terms of active transport of water by the cornea.

I would like to ask Dr. Harris several questions. Instead of using 95-percent oxygen and five-percent CO_2 in the moist atmospheres above the intact eye, did he ever try using only five-percent CO_2 and air?

Secondly, does he think it is necessary to have this high concentration of oxygen in order to maintain the sodium, water, and potassium content of cornea?

I would also like to ask Dr. Harris if he thought there was any shift in the pH of the aqueous resulting from accumulation of lactic acid when using the intact eye after a period of, say, 24 or 48 hours, and whether any such shift would affect his results?

Dr. V. Everett Kinsey (Detroit, Michigan): I would like to ask Dr. Harris to comment on a statement made several years ago by Dr. Maurice

who, in discussing the question of active transport of water or ions from the cornea, pointed out that the least efficient of the various mechanisms would be one in which water is actively transported.

What assumptions go into the calculation of the concentrations of various ions in the cornea with regard to the so-called sodium space?

DR. JOHN E. HARRIS (in closing): In regard to Dr. Schwartz' question of the oxygen concentration, I do not know the effect of oxygen at the partial pressure of the atmosphere. Presumably, it would have a metabolic effect. The point is worthwhile studying as at least a partial answer to the question of whether the cornea normally obtains some of its oxygen from the atmosphere.

In regard to the pH of the aqueous, certainly we do expect a shift in the aqueous pH during that time.

Regarding Dr. Kinsey's remarks, Dr. Maurice discussed various mechanisms which might operate across the barriers we are speaking of to maintain the normal hydration of the cornea. These possibilities included the active transport from the cornea of sodium or of water, among others. He pointed out that any living barrier is generally more permeable to water than to any other substance. Since the water turnover across it would be greater than the turnover of ions, a water pump would presumably be less efficient than an ion pump in maintaining normal corneal hydration. Such reasoning is logical but does not, of course, preclude the possibility. I might add, too, that a more rapid turnover of water is not a priori proof that a water pump requires more energy.

I think that much of the published work in the swelling of the cornea can be interpreted as indicating a pump of water rather than of an ion. Time does not permit a complete analysis. I am thinking though of some of your experiments, Dr. Kinsey, with Dr. Cogan, in which you measured the swelling of the cornea at various osmotic pressures. We do not definitely exclude the possibility of an ion transport.

In calculating the total base (more specifically the sum of sodium and potassium) concentration of the extracellular compartment one must assume a value for the cellular constituents. (Others have assumed as a first approximation that the corneal cells are devoid of sodium. This is hazardous. Epidermis, for example, has a fairly high sodium concentration.) The figure most commonly given for total base of cells is around 190 mEq. per 1,000 gm. of water. Human epidermis, for example, is in that range. The total base of the cells of the cornea is probably of similar magnitude. Since the sum of the sodium and potassium concentrations of the whole cornea is in that range, the assumption seems reasonable that the total base concentration in the extracellular compartment is similar to that of the whole cornea (in the unaltered state, of course). Thus, the figure 186 mEq. per 1,000 gm. of water. Our analyses showed a little more sodium than did Davson's. He found, as I recall, around 149, we found around 155, mEq. per 1,000 gm. of water.

SOME BIOCHEMICAL CHARACTERISTICS OF ACID INJURY OF THE CORNEA*

I. ASCORBIC ACID STUDIES

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In 1936 Henkes[1] reported an average concentration for ascorbic acid in the normal rabbit cornea of 18 to 20 mg. per 100 gm. of tissue. However, in more recent studies, Pirie[2] found the average concentration of ascorbic acid to be 55 mg. per 100 gm. normal rabbit cornea. The epithelium contained an average of 118 mg. per 100 gm. of tissue while the stroma contained 25 mg. per 100 gm. of tissue.

Campbell and Ferguson[3] in studying the role of ascorbic acid in corneal vascularization reported that new vessel invasion of the cornea following heat injury in the guinea-pig occurred with significantly greater frequency in scorbutic animals than in the control group. In a separate report of the same year Campbell[4] presented data which suggested that although restoration of corneal epithelium as such might be independent of an adequate supply of ascorbic acid, the rate of epithelization of a wound of the cornea involving collagenous tissue depended on the provision of a suitable fibrous substratum and in turn on an adequate intake of ascorbic acid.

In his studies of the chemistry of aqueous

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humor in glaucoma, Kronfeld[5] reported that ascorbic acid level of the aqueous was dependent upon the blood-ascorbic acid level in the normal eye as well as in the eye affected with deep-chamber glaucoma. Kinsey[6] observed that, above a certain critical plasma level further increase in the ascorbic acid content of the blood did not significantly increase the ascorbic acid content of the aqueous.

The rate of turnover of ascorbic acid in the aqueous has been determined by several investigators. A value of 0.8 percent per minute was reported by Friedenwald and co-workers.[7] Kinsey and Bárány[8] working under more physiologic conditions reported a turnover rate of one percent per minute. Using still a different method, Becker and co-workers[9] reported a value of 1.3 to 1.5 percent per minute.

Kinsey[10] has shown that the aqueous humor of the posterior chamber contains one and one-half times as much ascorbic acid as that in the anterior chamber.

It was the object of the present work to determine:

a. The effect of hydrochloric acid injury on the ascorbic acid content of the cornea immediately after injury and during the course of recovery.

b. The effect of this acid injury on the ascorbic acid content of aqueous humor immediately after injury and during the course of repair.

c. The direct cause of any change in ascorbic acid concentration at these two sites.

Methods

Assay of ascorbic acid

Corneas were prepared for ascorbic acid assay by extraction with 20-percent metaphosphoric acid for one hour. Aqueous humor samples also were placed in this extraction medium immediately after procurement and allowed to stand for one hour. Protein free aliquots from these extracts were used to assay ascorbic acid.

The amount of ascorbic acid in the extracts was determined by titration with 2,6-dichlorophenolindophenol using an ultramicroburette.

The 2,6-dichlorophenolindophenol was standardized by titration with pure ascorbic acid solution. The ascorbic acid solution was standardized against an iodine-potassium iodide standard of known oxidation-reduction normality.

Nitrogen determination. Nitrogen determinations were made by utilizing the Kjeldahl digestion technique for the conversion of nitrogen to ammonia and after proper dilution the ammonia was determined colorimetrically by the method of Borsook.[11]

Lactate determinations. The Barker-Summerson[12] method of lactate determination was used.

Anterior chamber aqueous samples. Samples of aqueous humor from the anterior chamber were obtained by puncturing the cornea with a No. 26-gauge needle and drawing the aqueous (usually 0.05-0.1 ml.) into a 0.2-ml. syringe.

Posterior chamber aqueous samples. Aqueous humor samples from the posterior chamber were obtained with a micropipette designed by Kinsey[10] using the technique described by the same author in 1953.[13]

Animals. The animals used in this work were albino rabbits of the New Zealand White strain weighing from 1,500 to 2,500 gm. They were maintained on a diet of Gibbons Rabbit Pellets supplemented with approximately one-fourth pound of raw cabbage per day. Water was supplied freely.

Standard burning technique. The hydrochloric acid injury was applied to the right eye of each experimental animal leaving the left eye as a control. The rabbits were placed in a special box which held their heads rigid. The lids were held open with a speculum and the nictitating membrane was retracted with a Desmarres retractor. Hydrochloric acid of 1.0N strength was dropped on the cornea from a standardized pipette at the rate of two drops every 10 seconds for one minute. The eye then was washed with distilled water for 15 seconds.

Statistical treatment. The data obtained

were submitted to analysis by the Fisher "T" test of significance. The p values were obtained from *Statistical Tables for Biological, Medical, and Agriculture Research* by Fisher and Yates.[14]

Results

Corneal ascorbic acid

In these experiments the whole corneas were extracted for ascorbic acid assay, after which the nitrogen content of the tissue was determined. The values of corneal ascorbic acid were expressed in terms of micrograms ascorbic acid per mg. nitrogen.

A considerable amount of difference was found in the concentration of ascorbic acid in the normal corneas of different rabbits, but the difference in ascorbic acid concentration in the two normal corneas from the same animal was found to be rather small (table 1). There was no significant difference between the right and left eye values in this set of animals.

The standard burning technique was applied to the right eyes of a group of rabbits. At various time intervals after onset of injury the animals were sacrificed by air injection into the marginal ear vein and the corneas removed. Ascorbic acid assay and nitrogen determinations were performed on the corneas.

The ascorbic acid content of corneas injured by hydrochloric acid decreased immediately after application of the burn and remained significantly lower than the content of the normal controls for approximately 120 hours after onset of injury (table 2). After 120 hours there no longer was any significant difference between the burned and normal corneas.

TABLE 1

Comparison of ascorbic acid concentrations in normal corneas of rabbits

Animal No.	μg. Ascorbic Acid/mg. N		R.E./L.E. Percent	T	P
	Right Eye	Left Eye			
1	13.5	14.9	91		
2	14.0	14.1	99		
3	15.3	15.4	99	0.44	0.8–0.7
4	17.8	17.0	105		

TABLE 2

Ascorbic acid concentration in burned and normal corneas at various time intervals after onset of injury

Time After Onset of Injury	Exp. No.	μg. Ascorbic Acid/mg. N		T	P
		Right Eye	Left Eye		
0	1	5.7	9.9		
	2	4.2	8.4	7.59	0.01–0.001
	3	4.5	6.7		
	4	8.8	12.8		
24	1	6.5	13.6		
	2	6.3	13.3	10.53	0.01–0.001
	3	3.4	10.9		
	4	5.1	15.3		
48	1	4.3	12.1		
	2	4.5	13.7	12.70	0.01–0.001
	3	5.8	13.2		
	4	5.7	16.1		
96	1	5.9	9.9		
	2	4.2	15.2	4.69	0.02–0.01
	3	5.8	15.4		
	4	4.3	18.0		
120	1	5.1	11.2		
	2	7.8	12.6	3.78	0.05–0.02
	3	11.0	13.0		
	4	9.1	11.3		
168	1	21.5	22.4		
	2	31.9	28.8	1.28	0.30–0.20
	3	9.8	17.5		
	4	9.9	19.6		

These data were expressed as the ratio of ascorbic acid content of the burned cornea to that of the normal cornea from the same rabbit (fig. 1).

The question of whether the ascorbic acid itself was destroyed by the hydrochloric acid burn was investigated. To determine this the following experiment was performed.

Normal rabbits were killed by air injection and both normal corneas removed and placed in dry test tubes. To the right corneas 14 drops of 1.0N. hydrochloric acid were added over a period of one minute. Similarly 14 drops of distilled water were added to the left corneas. Twenty-percent metaphosphoric acid was added to all tubes and after one hour the extracts were assayed for ascorbic acid and nitrogen determinations made on the corneas. No significant difference in the ascorbic acid value of burned and normal corneas was found (table 3).

Aqueous humor ascorbic acid

The effect of this injury on the ascorbic acid concentration of the aqueous of the anterior chamber was determined.

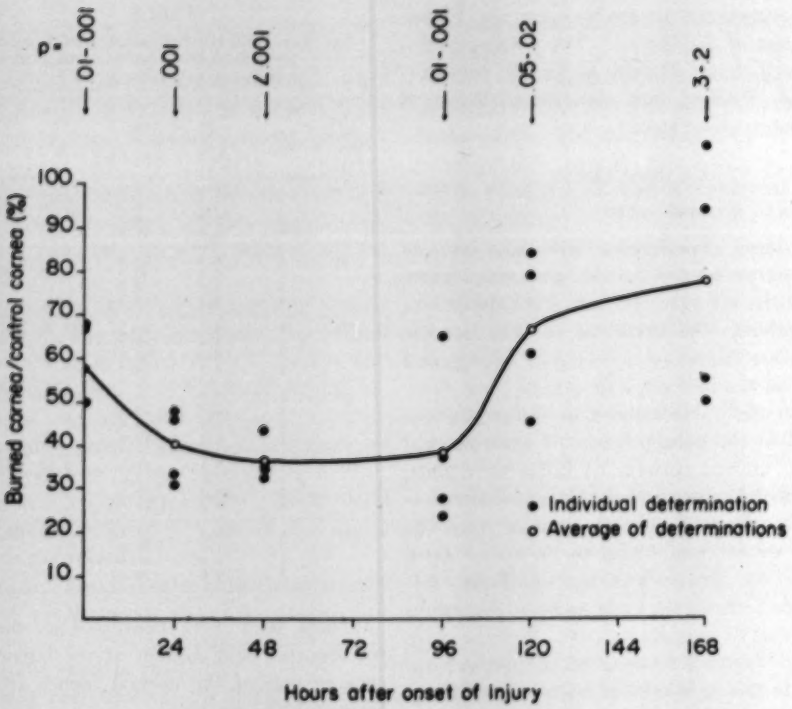

Fig. 1 (Guidry, Allen, and Kelly). Ratio of ascorbic acid content of the burned cornea to that of the normal cornea from the same rabbit.

A considerable amount of difference was found in the concentration of ascorbic acid in the normal anterior aqueous of different rabbits, but the difference in concentration in the two normal eyes from the same animal was found to be rather small (table 4).* There was no significant difference between the right and left eye values in this set of animals.

The right eyes of a group of rabbits were burned by the standard burning technique and at various time intervals after onset of injury the animals were killed by air injection. Samples of anterior chamber aqueous humor were immediately obtained and flushed into 20-percent metaphosphoric acid. Ascorbic acid was assayed by titration and the values from the burned eyes compared to the control eyes. There was no immediate effect on the aqueous humor ascorbic acid

TABLE 3

IMMEDIATE EFFECT OF BURN ON CORNEAL ASCORBIC ACID

Animal No.	μg. Ascorbic Acid/mg. N		R.E./L.E. Percent	T	P
	Right Eye	Left Eye			
1	9.3	9.9	94		
2	13.0	15.0	87		
3	9.2	11.9	77		
4	9.2	7.9	116	1.77	0.2-0.1
5	10.3	10.2	101		
6	6.0	7.3	82		
7	5.8	7.2	81		

* The aqueous ascorbic acid values obtained in these experiments are higher than usually reported; however, the blood plasma ascorbic acid levels were higher than usual in this series of rabbits, probably because of the dietary supplementation with cabbage. The average plasma ascorbic acid level was 2.7 mg. percent with a range of 2.07 to 3.32 mg. percent. Similar rabbits without cabbage added to the basic diet averaged approximately 1.0 mg. percent ascorbic acid plasma levels.

TABLE 4

COMPARISON OF ASCORBIC ACID CONCENTRATION IN NORMAL ANTERIOR CHAMBER AQUEOUS OF RABBITS

Animal No.	μg. Ascorbic Acid/100 ml Aqueous		R.E./L.E. Percent	T	P
	Right Eye	Left Eye			
1	49.1	40.9	120		
2	49.0	51.5	95		
3	51.5	48.5	106		
4	34.4	32.0	108	1.29	0.3-0.2

content by the hydrochloric acid injury (table 5). Twenty-four hours after onset of injury, however, there was a significant decrease in aqueous ascorbic acid concentration, and this decrease persisted for approximately 120 to 144 hours after onset of injury.

These data were expressed as the ratio of ascorbic acid content of the anterior aqueous of the burned eye to that of the normal eye from the same rabbit (fig. 2).

TABLE 5

ASCORBIC ACID CONCENTRATION IN ANTERIOR CHAMBER AQUEOUS OF BURNED AND NORMAL RABBIT EYES

Time After Onset of Injury	Exp. No.	μg. Ascorbic Acid/100 ml. Aqueous		R.E./L.E. Percent	T	P
		Right Eye	Left Eye			
(hrs.)						
0	1	55.8	60.4	92		
	2	81.5	78.0	104	0.26	0.9 -0.8
	3	71.6	69.7	103		
	4	69.9	72.8	96		
24	1	19.2	29.6	65		
	2	15.4	21.0	73		
	3	26.3	36.0	73	5.30	0.01-0.001
	4	20.4	35.4	58		
48	1	30.3	41.4	73		
	2	36.6	52.9	69	7.43	0.01-0.001
	3	10.1	23.4	43		
	4	16.3	24.8	66		
72	1	19.9	34.2	58		
	2	18.9	34.0	56	11.62	0.01-0.001
	3	25.5	46.3	55		
	4	18.3	35.6	51		
96	1	30.3	47.5	64		
	2	30.7	53.4	57	6.85	0.01-0.001
	3	12.9	24.1	54		
	4	22.8	44.9	51		
120	1	40.9	44.7	91		
	2	23.1	27.2	85	30.00	0.001
	3	42.9	46.9	91		
	4	37.3	40.8	91		
144	1	37.2	48.7	76		
	2	37.4	38.2	98	2.00	0.1 -0.05
	3	29.0	30.5	95		
	4	20.8	26.1	80		
168	1	45.9	51.0	90		
	2	49.2	51.9	95	2.67	0.2 -0.1
	3	38.7	49.0	79		

UTILIZATION OF ASCORBIC ACID BY BURNED CORNEAS

Both eyes of a group of rabbits were burned by the standard burning techniques. At several time intervals after onset of injury (corresponding to time periods when aqueous ascorbate concentration was at its lowest) the corneas were removed after the animals were killed. They were placed in 9×10^{-3}M phosphate buffer of pH 7.0 containing ascorbic acid. The buffer was made isotonic by the addition of sodium chloride. Some of the corneas were placed in ascorbic acid-free buffer as controls to determine the amount of this substance which could be washed out of the cornea into the buffer during the period of incubation. A control flask containing buffer and ascorbic acid but no corneas was also incubated to determine the amount of ascorbic acid spontaneously destroyed during the experiment.

All of the flasks were incubated at 37°C. for one hour with shaking, after which aliquots of the suspending media were taken and assayed for ascorbic acid. No significant utilization of ascorbic acid by burned corneas was demonstrated (table 6).

UTILIZATION OF ASCORBIC ACID BY BURNED IRISES

Experiments identical with those just described for burned corneas, with the exception that irises were substituted for corneas, were performed. No significant utilization of ascorbic acid by these irises was demonstrated with the technique used (table 7).

TABLE 6

UTILIZATION OF ASCORBIC ACID BY BURNED CORNEAS

Hr. After Burn	Ascorbic Acid in Media After Incubation (Control)	Ascorbic Acid Spilled into Media	Total Ascorbic Acid in Media	Ascorbic Acid in Media After Incubation with Tissue
48	369 μg.	17* μg.	386 μg.	432* μg. (12%)
72	388 μg.	3* μg.	391 μg.	422* μg. (8%)
96	480 μg.	0* μg.	480 μg.	488* μg. (2%)

* Average of two determinations.

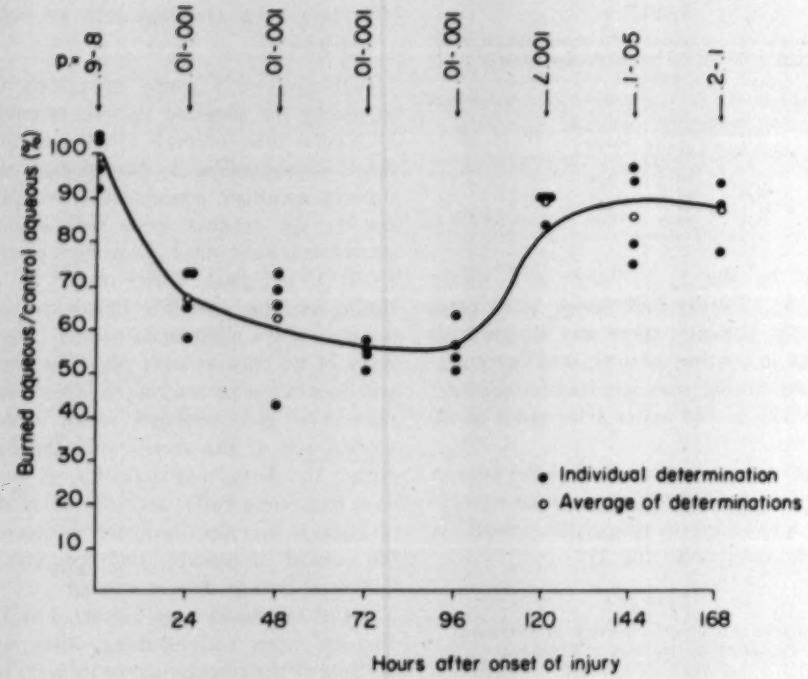

Fig. 2 (Guidry, Allen, and Kelly). Ratio of ascorbic acid content of the anterior aqueous of the burned eye to that of the normal eye from the same rabbit.

Effect of acid burns of cornea on posterior/anterior aqueous humor ascorbic acid ratios

Considerable variation in the ratios of the concentration of ascorbic acid in the posterior chamber aqueous to that of the anterior chamber aqueous of normal eyes from different rabbits was observed (table 8). However, there was no significant difference in these ratios for the two normal eyes of the same rabbit in the group studied.

TABLE 7

Utilization of ascorbic acid by irises from burned eyes

Hr. After Burn	Ascorbic Acid in Media After Incubation (Control)	Ascorbic Acid Spilled into Media	Total Ascorbic Acid in Media	Ascorbic Acid in Media After Incubation with Tissue
48	369 μg.	17* μg.	386 μg.	434* μg. (12%)
72	388 μg.	5* μg.	393 μg.	433* μg. (10%)
96	480 μg.	0* μg.	480 μg.	488* μg. (2%)

* Average of two determinations.

The right eyes of a group of rabbits were burned by the standard burning technique. At various time intervals after onset of injury, the aqueous samples from the posterior and anterior chambers were removed, and assayed for ascorbic acid.

The ascorbic acid content of the aqueous of the posterior chamber of the burned

TABLE 8

The ratios of posterior chamber aqueous ascorbic acid to anterior chamber aqueous ascorbic acid on normal rabbits

Exp. No.	Ratio of Ascorbic Acid in Posterior Chamber to Anterior Chamber		T	P
	Right Eye	Left Eye		
1	1.72	1.67		
2	1.45	1.63	0.35	0.8-0.7
3	1.40	1.40		
4	1.30	1.25		

TABLE 9

COMPARISON OF ASCORBIC ACID CONCENTRATION OF POSTERIOR CHAMBER AQUEOUS IN BURNED AND NORMAL EYES

Time After Onset of Injury (hrs.)	Exp. No.	Ascorbic Acid μg./100 ml. Aqueous Right Eye	Left Eye	T	P
24	1	71.7	75.5		
	2	45.8	50.6	1.98	0.2 -0.1
	3	65.7	66.4		
	4	66.9	81.8		
48	1	60.5	46.7		
	2	46.0	61.3		
	3	65.2	61.0	0.20	0.9 -0.8
	4	67.8	75.5		
72	1	45.0	49.8		
	2	37.5	55.6	3.82	0.05-0.02
	3	40.7	54.0		
	4	35.1	43.4		

eyes was not significantly different from that of the normal eyes at 24 and 48 hours (table 9). There was, however, a decrease in posterior aqueous concentration at 72 hours after onset of injury. The significance of this difference is borderline and, due to the great difficulty in obtaining posterior aqueous in the burned eyes at this time period, it is highly probable that this difference is insignificant.

The ratio of ascorbic acid content of the aqueous of the posterior chamber to that of the anterior chamber of the burned eye was significantly higher than the ratios in the normal eye (table 10) for the time periods 24, 48, and 72 hours.

TABLE 10

RATIO OF CONCENTRATION OF ASCORBIC ACID IN POSTERIOR CHAMBER AQUEOUS TO THAT IN ANTERIOR CHAMBER AQUEOUS FOR BURNED AND NORMAL EYES AT THREE TIME INTERVALS AFTER ONSET OF INJURY

Time After Onset of Injury (hrs.)	Exp. No.	Ratio of Concentration of Ascorbic Acid in Posterior Chamber/Anterior Chamber Right Eye	Left Eye	T	P
24	1	1.63	1.15		
	2	1.90	1.47	9.29	0.01-0.001
	3	1.93	1.65		
	4	2.03	1.67		
48	1	1.52	1.13		
	2	1.82	1.26	7.63	0.01-0.001
	3	2.06	1.33		
	4	2.10	1.36		
72	1	1.62	1.10		
	2	1.63	1.17	10.95	0.01-0.001
	3	1.63	1.28		
	4	1.92	1.42		

AQUEOUS HUMOR LACTATE

The effect of hydrochloric acid injury of the cornea on the concentration of lactic acid in the anterior aqueous was determined. The right eyes of a group of rabbits were burned as previously described and the animals were sacrificed by air injection at various time intervals after onset of injury. Anterior aqueous samples were obtained and the lactate content determined. There was a significant decrease in the lactate content of the anterior aqueous in those eyes which were injured by the acid (fig. 3). The points at each time interval are averages of four determinations. This decrease in the lactate concentration paralleled the decrease in anterior chamber aqueous ascorbic acid content.

DISCUSSION

The ascorbic acid content of the cornea of rabbit eyes injured by hydrochloric acid was markedly decreased immediately after

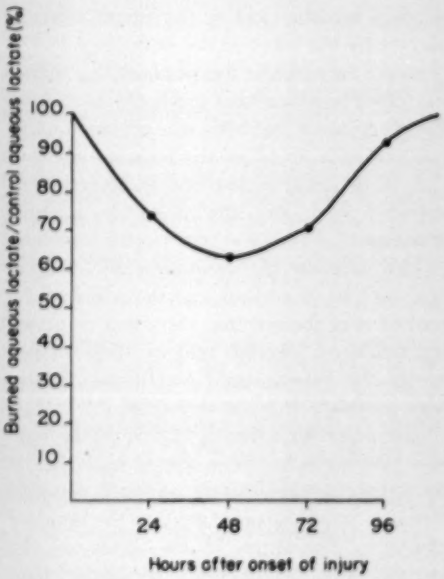

Fig. 3 (Guidry, Allen, and Kelly). Concentration of lactate in anterior-chamber aqueous of burned eyes at various time intervals after onset of injury. (All points represent averages of determinations on four animals.)

the application of the injury. This concentration remained lower than normal for the first few days after onset of injury and slowly increased as the cornea healed. The ascorbic acid itself was not destroyed by the injury, but apparently was released from the cornea by the acid and was washed away by the acid and the wash water used during the application of the injury.

The ascorbic acid concentration of the anterior aqueous was not immediately affected by the injury, but 24 hours after onset of injury this concentration decreased significantly and remained low for several days. Then the concentration increased and normal values were obtained at approximately the same time that the concentration of ascorbic acid in the cornea reached a normal value.

Several postulates were formed to explain the decrease in anterior aqueous humor ascorbic acid. These were:

a. Abnormally rapid utilization of the aqueous ascorbic acid by the burned corneas and/or by the irises of the burned eye.

b. An increase in the permeability of the iris. This postulate was prompted in part by the observation that iritis was observed without exception in all of the burned eyes.

c. A decrease in the rate of secretion of ascorbic acid into the posterior aqueous chamber.

The data on the utilization of ascorbic acid by burned corneas and the irises of the burned eyes showed that there was no actual utilization of ascorbic acid by these tissues under the experimental conditions. Therefore postulate (a) was untenable.

The posterior aqueous humor of the normal rabbit eye has been shown by Kinsey[10] to contain approximately one and one-half times as much ascorbic acid as the anterior chamber. Therefore, if the irises of the burned eyes became more permeable than those of the normal eyes thus allowing an increase in the rate of escape of ascorbic acid from the anterior chamber, and if the secretion of ascorbic acid into the posterior chamber were unaffected, one would expect an increase in the ratio of the concentrations of ascorbic acid in the posterior chamber to that in the anterior chamber. The data obtained supports this hypothesis.

It was further shown that there was no significant difference in the concentration of ascorbic acid in the posterior chambers of the burned and normal eyes indicating that the secretion of ascorbic acid was not significantly influenced by the burn.

The lactate of aqueous humor is believed to be derived from the lens. It is thought that this substance enters the aqueous in the posterior chamber and is carried into the anterior chamber by normal aqueous flow. The decrease in concentration of lactate in the anterior aqueous in the burned eyes also supports the hypothesis that permeability of the iris vessels was increased as a result of the burn.

Summary

1. The ascorbic acid content of rabbit cornea is significantly decreased immediately after injury by hydrochloric acid. The ascorbic acid itself is apparently not destroyed, but it is released from its bound sites in the cornea and is washed away during the burning procedure.

2. The ascorbic acid concentration in the anterior aqueous humor is not immediately affected by the injury, but a decrease in concentration occurs 24 hours after onset of injury. Data have been presented which suggest that the immediate cause of the decrease in anterior chamber ascorbic acid is an increase in the permeability of the iris vessels. This is apparently due to the iritis which is always observed in the burned eye. No signicant difference in the rate of secretion of ascorbic acid into the posterior chamber was found in the burned and normal eyes.

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Acknowledgement

The authors wish to acknowledge with thanks the helpful suggestions of Dr. Bernard Becker and V. Everett Kinsey on this study.

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Discussion

Dr. Jonas S. Friedenwald (Baltimore, Maryland): It is gratifying when, in a study of this sort, reasonable expectations are fulfilled. Trauma to the cornea of sufficient severity should affect the blood-aqueous barrier, rendering it more permeable. Ascorbic acid, normally present in the aqueous in great excess over its plasma concentration, should diffuse out with greater facility, and the concentration in the anterior chamber should fall. The authors find the aqueous-ascorbic concentration at a low level following the injury, as one would expect.

Unhappily, they have done only half of the job in this study. They have shown that the expected event occurred; they have not shown that it occurred by the expected mechanism.

A complete study of this phenomenon should include two other features. If the blood-aqueous barrier is broken by this injury, that can be tested directly by appropriate techniques, by an increase in the aqueous protein, or by the inulin test worked out many years ago by Swann and Hart.

In addition, there is need to consider the possibility that the rate of aqueous secretion might be diminished as a consequence of the corneal injury. Direct tests for the rate of aqueous flow are now available, and they need to be done in this experiment before the study can be said to be complete. If the aqueouses were stagnant, even if the blood-aqueous barrier in the anterior chamber is not altered, one would expect a decline of aqueous ascorbate. There are nowadays direct methods of measuring the rate of aqueous flow.

The notion that corneal injury may cause partial inhibition of aqueous-humor secretion is, in fact, supported by a good deal of indirect evidence. Recent studies by Kinsey, by Becker, and by myself show that the turnover rate of ascorbic acid in the undisturbed rabbit's eye is over 2.0 (somewhere between 2.0 and 2.5) percent per minute. Of this, roughly a half percent represents diffusional exchange, and something over 1.5 percent represents flow.

Many years ago, Dr. Buschke and I attempted to measure the turnover rate of ascorbic acid by the very naïve and simple procedure of injecting a large excess of ascorbic acid into the anterior chamber and observing the disappearance of the artificial excess at various times later. We did this injection with the maximum precaution not to irritate the rabbit's eye. We found that we could remove the aqueous 20 and 40 minutes after such an injection, and that it contained no excess protein, so that we had not seriously irritated the eye or broken the blood-aqueous barrier. What we found at that time was a turnover rate of less than 1.0 percent per minute.

Incidentally, the authors quote these contradictory findings between the older experiments and the more recent ones; and I think the only serious in-

terpretation which can be given to this difference is that the mere sticking of a needle through the cornea temporarily diminishes aqueous secretion. I would suspect that burning it with one normal strength hydrochloric acid might be equally effective.

At any rate, what I think is that the authors have opened an entertaining field, and I hope they will be encouraged to finish the job they have begun.

DR. M. A. GUIDRY (New Orleans): I want to thank Dr. Friedenwald for his kind remarks and suggestions. We know that the work is only half done; I only wish I could convince myself that it is half done.

There are experiments now in progress which we hope will yield information toward the goal of finding out exactly if this blood-aqueous barrier has been broken, and what the direct cause of this break might be.

LENS-INDUCED ENDOPHTHALMITIS*

EXPERIMENTAL BILATERAL ENDOPHTHALMITIS PHACOANAPHYLACTICA

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The purpose of the experimental study to be reported was to determine (1) if a bilateral endophthalmitis phacoanaphylactica, similar to that occurring in human subjects,[1-3] could be produced in noncataractous rabbits by injuring first one lens and then the other, and (2), if possible, to establish the essential factors needed for the development of the process.

Lens sensitivity studies in animals have been reported by a number of investigators, notably by Verhoeff and Lemoine,[4] Burky and Woods,[5] Burky and Henton,[6] and by Müller.[7] Burky and Henton[6] successfully sensitized rabbits to lens antigen by the subcutaneous injection of lens material and an adjuvant of prepared staphylococcus toxin. When the eyes of these systemically sensitized animals were traumatized by rupturing the lens, a reaction claimed to be similar to endophthalmitis phacoanaphylactica was produced. In subsequent studies these authors were able to demonstrate positive skin tests to lens antigen, as well as high precipitin titers in the serum of their animals.

Our intent in the present study was to parallel in rabbits the sequence of events that are reported to lead to the development of bilateral endophthalmitis phacoanaphylactica in humans. It was proposed to sensitize the animals to lens antigen systemically by injecting an adjuvant into the traumatized lenses, rather than to use the subcutaneous injection of lens material and an adjuvant described by Burky and Henton.[6]

PROCEDURE

There were 40 young adult albino rabbits weighing 2.0 to 2.5 kg. used in the experiment. The rabbits were divided into groupings for the four phases of the study, four rabbits being reserved as controls.

In the first phase of the experiment eight rabbits were systemically sensitized to ox serum by the method of Schlaegel and Davis.[8] Subcutaneous injections of ox serum were administered at five-day intervals until a highly positive Arthus phenomenon was obtained following the fifth injection.

The lenses of the right eyes of the eight rabbits and of the four controls were punctured with a Ziegler knife and the lens material agitated into the anterior chamber. Clinically, there was a minimal inflammatory response in the eyes that did not differ from the response observed in the controls. A slight conjunctival hyperemia and cloudiness of the anterior chamber from the dispersed

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lens material was the only response noted. This reaction had completely subsided by 72 hours.

Three intradermal injections of lens antigen, given at three- to five-day intervals were negative.

At two weeks, discission of the posterior lens capsule of the left eye was done by puncturing the posterior sclera with a Zeigler knife and then scratching the lens capsule posteriorly[9] (fig. 1).

Following the discission into the posterior capsule there was a mild response similar to that seen in the right eyes. This reaction also subsided within 72 hours.

Histologic sections of both eyes of the serially killed animals showed a minimal cellular response which had disappeared by four days. A few pseudoeosinophilic polymorphonuclear leukocytes were noted near the ciliary body and a few mononuclear cells were observed clustered near the anterior retina. The only other finding was a relative vasodilatation of the vessels of the ciliary body and iris (fig. 2).

In the second phase of the experiment eight rabbits were systemically sensitized by the same method as were the animals in the first group. At the time of the discission of the right lenses 0.2 cc. of a commercially prepared solution of staphylococcus toxin was injected into the dispersed lens material as an adjuvant.

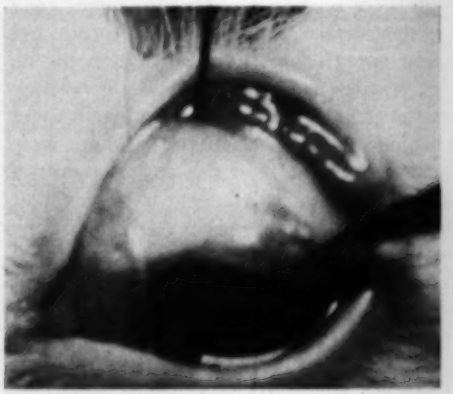

Fig. 1 (Lyda and Lippincott). Rupturing the posterior lens capsule by passing a Graefe knife through the sclera behind the equator.

Fig. 2 (Lyda and Lippincott). Histologic section of the right eye of a rabbit sensitized to ox serum and a discission of the lens done.

The clinical response in this group did not differ from that found in the first group or in the controls. A minimal conjunctival hyperemia incidental to the trauma was seen but it subsided within 72 hours (fig 3).

There was a negative response to the three skin tests to lens antigen in this group.

At two weeks a posterior discission of the lenses of the left eyes was done. This procedure was also followed by a minimal conjunctival hyperemia that cleared within 72 hours.

Histologic sections of both eyes of the serially killed animals showed findings similar to those seen in the first group. There were a few leukocytes and mononuclear cells

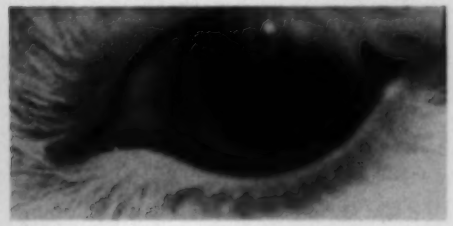

Fig. 3 (Lyda and Lippincott). The appearance of the right eye of an ox-serum sensitized rabbit after discission and injection of staphylococcus toxin into the lens.

scattered in the region of the ciliary body, a slight degree of vasodilatation, and a dispersion of lens material into the vitreous (fig. 4).

In the third phase of the study four rabbits were systemically sensitized to staphylococcus toxin by the intravenous injection of 1.5 cc. of a commercially prepared solution. At 24 hours a skin test to the staphylococcus toxin was considered to be a two-plus to three-plus reaction. A simple discission into the right lenses was then made.

The resulting response consisted of a mild conjunctival hyperemia and ciliary blush that increased slightly during the first 24 hours but disappeared at 72 hours.

Skin tests to lens antigen were negative on three occasions.

At two weeks a posterior discission of the left lenses was performed. There was again a mild response that cleared within 72 hours.

Histologic sections of the right eyes showed no signs of inflammation or vasodilatation. The left eyes revealed a few leukocytes and mononuclear cells infiltrating from the region of the ciliary body.

In the final phase of the experiment 16 rabbits were systemically sensitized to staphylococcus toxin by the intravenous injection of 1.5 cc. of a prepared solution. After a 48-hour waiting period the animals were skin tested by the intradermal injection of 0.1 cc. of the same preparation. When the tests were read at 24 hours they were interpreted as being a three-plus response and indicative of an adequate sensitization.

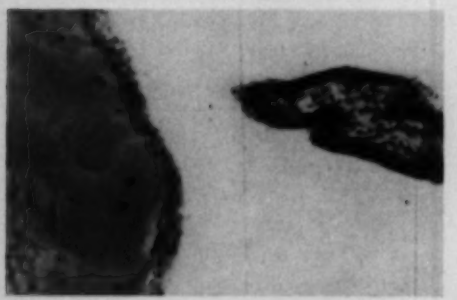

Fig. 4 (Lyda and Lippincott). Histologic section of the right eye of a rabbit sensitized to ox serum and staphylococcus toxin injected into the eye at the time of the discission. There is very little reaction present.

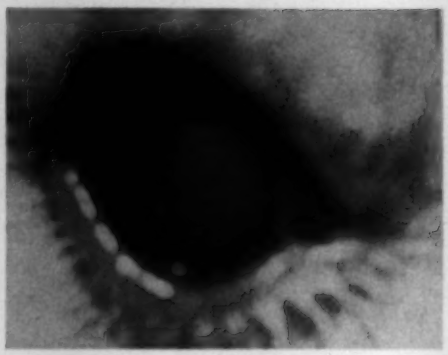

Fig. 5 (Lyda and Lippincott). The appearance of the right eye 24 hours following discission and injection of staphylococcus toxin in a staphylococcus sensitized rabbit.

A discission of the right lenses was performed and 0.2 cc. of the staphylococcus toxin was injected into the dispersed lens material.

Clinical observation of the eyes during the first 24 hours revealed a rather marked inflammatory reaction following the discission and injection of the staphylococcus toxin. The cornea became cloudy in its central portion. There was a well-developed ciliary blush and conjunctival hyperemia. Many cells were seen in the anterior chamber and on the surface of the posterior cornea and there appeared to be vasodilatation of the iris vessels (fig. 5). At 48 hours the reaction was even more marked. The conjunctiva was chemotic as well as hyperemic. There were heavy precipitates on the posterior cornea and the cornea had become so cloudy that the iris could not be seen. At 72 hours the severity of the reaction remained unchanged (fig. 6). At five days there was some clearing of the cornea so that a fairly marked dilatation of the iris vessels could be seen as well as precipitates on the posterior cornea. The conjunctival hyperemia and ciliary blush remained about the same. At eight days the ciliary blush was still intense but most of the chemosis had subsided. A membranelike

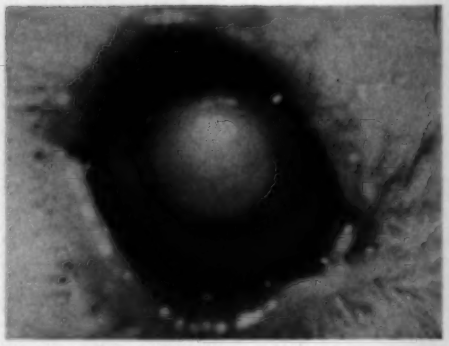

Fig. 6 (Lyda and Lippincott). The appearance of the right eye 72 hours following discission and injection of staphylococcus toxin in a staphylococcus sensitized rabbit.

structure could be seen over portions of the disturbed lens material.

An intradermal skin test to lens antigen was made 48 hours following the discission and injection of staphylococcus toxin into the right eyes. When the skin tests were read after 24 hours there was slight increase in the skin reactions as compared to the controls. At five days the test was repeated and at the end of 24 hours a one-plus to two-plus response, as judged by Burky and Woods[8] criteria, was found (fig. 7). At eight days the test was repeated for the third time and an average response of two-plus was found after 24 hours (fig. 8).

Following the final skin test to lens antigen and eight days following the trauma to the right eyes, a discission was made through the posterior lens capsules of the left eyes with a Ziegler knife. Within 24 hours there was a moderate inflammatory response as evidenced by a conjunctival hyperemia, ciliary blush and corneal haziness. This reaction became progressively more severe so that at 48 hours there was a marked chemosis and a cornea so cloudy that the iris could not be seen clearly.

It was interesting to note that at this time there was a resurgence of the inflammatory response in the right eyes as evidenced by a more intense conjunctival injection and anterior chamber cloudiness.

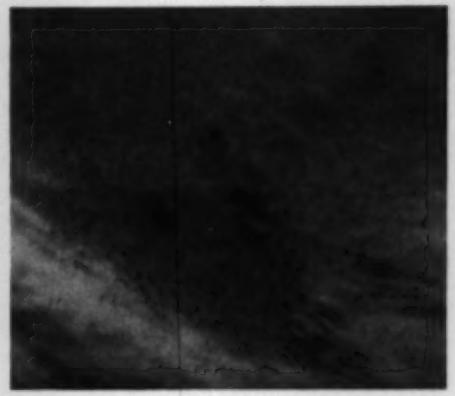

Fig. 7 (Lyda and Lippincott). A positive skin reaction to lens antigen in a rabbit systemically sensitized to staphylococcus toxin and the toxin injected into the lens substance.

At 72 hours the reaction was still more severe in the left eye but after the fourth day the process gradually subsided, the left eyes reaching a quiescent state before the right eyes at the 21st day of observation.

Histologic sections of the right eyes of rabbits killed 24 hours after discission of the left eyes showed a rupture of the lens capsule and beginning proliferation of the lens epithelial cells. There was an infiltration of the iris and ciliary body with numerous leukocytes. Nests of these cells could be seen in the lens substance. Near the retina there was an accumulation of mononuclear cells. A moderate dilatation of the vessels of

Fig. 8 (Lyda and Lippincott). A second positive skin test to lens antigen in a rabbit systematically sensitized to staphylococcus toxin and the toxin injected into the lens substance.

the choroid, iris, and ciliary body could be seen (fig. 9). In the left eyes there was a similar response with a preponderance of leukocytes, scattered mononuclear cells, and an edema of the ciliary body and choroid being found.

At 48 hours the histologic studies of the right eyes showed an intensity of response similar to that found in the first 24 hours. In addition, many of the leukocytes seemed to have ingested large amounts of lens material. These cells appeared to have originated in the dilated vessels of the ciliary body and iris. The mononuclear cells tended to congregate nearer the retinal vessels and posterior ciliary body. The left eyes of the animals killed at 48 hours showed a response very similar to that observed in the right eyes, but less marked. There were fewer cells and less vasodilatation (fig. 10).

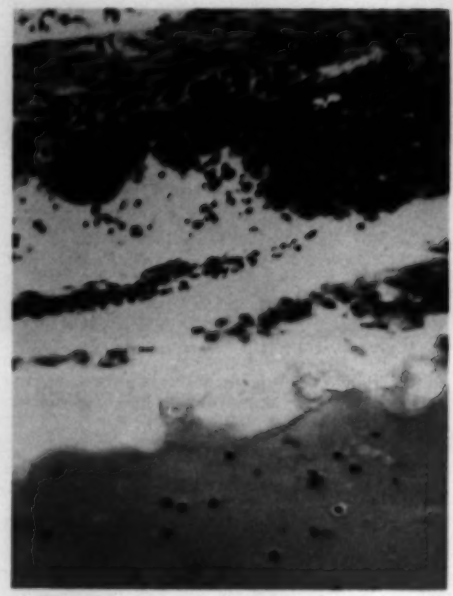

Fig. 10 (Lyda and Lippincott). Histologic section of the left eye of a rabbit killed 48 hours after discission. There is a moderate vasodilatation and cellular infiltration is present.

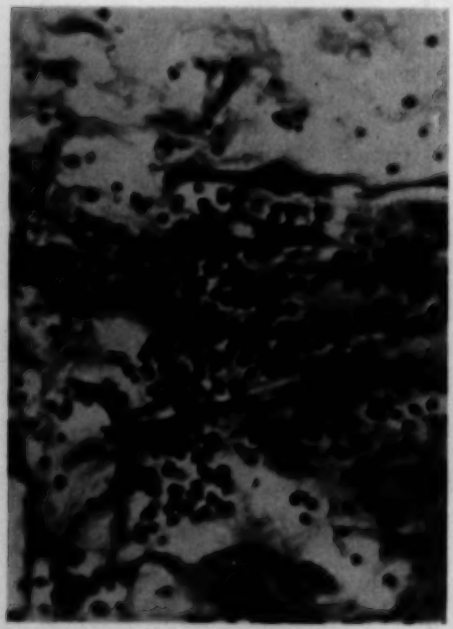

Fig. 9 (Lyda and Lippincott). Histologic section of the right eye of a rabbit killed 24 hours following discission of the left eye. There is an infiltration of the iris and the ciliary body and portions of the lens substance with polymorphonuclear leukocytes.

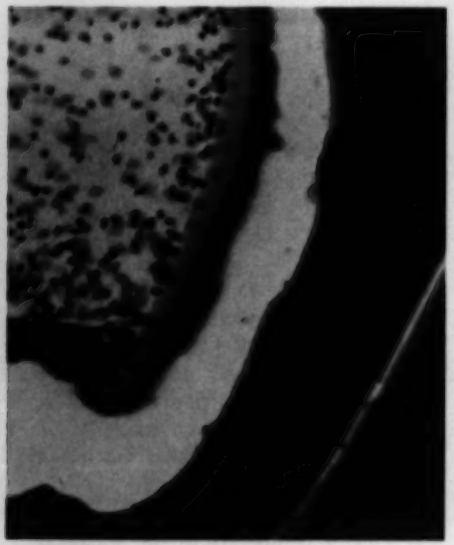

Fig. 11 (Lyda and Lippincott). Histologic section of the right eye of a rabbit killed 72 hours after discission of the left lens. There is an accumulation of dispersed cells in the lens material, some of which contain ingested lens material.

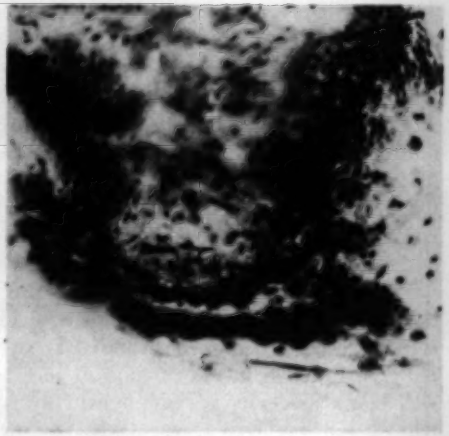

Fig. 12 (Lyda and Lippincott). The findings in the right eye of a rabbit killed four days following discission of the posterior capsule of the left lens. There are a few cells present but a significant vasodilatation of the uveal vessels persists.

The histologic sections of the right eyes of the animals killed at 72 hours revealed a less intense cellular response. The leukocytes tended to accumulate in the dispersed lens material with only a few of the cells being seen in the vitreous. Those cells located in the lens contained quantities of ingested lens substance (fig. 11). In the left eyes there were relatively more mononuclear cells and these also contained ingested lens material. The leukocytes tended to accumulate in the lens material and the mononuclear cells tended to accumulate nearer the retina.

The right eyes of the rabbits killed at four days had a lessening in the total cellular response. The predominant cell continued to be the leukocyte with only a few scattered mononuclear cells being found. Although there was a decrease in the cellular response, the edema and vasodilatation of the conjunctiva and uvea persisted (fig. 12). The left eyes of the animals had only a small number of leukocytes in the anterior vitreous and in the lens substance, but there was a significant degree of vasodilatation of the conjunctiva, iris, and ciliary body.

In the rabbits killed at five days, that clinically had a slight decrease in the inflammatory response, the histologic sections of the right eyes revealed small groups of leukocytes and a few mononuclear cells. In the left eyes there was a diffuse spread of amorphous lens material in a vitreous containing a few scattered cells (fig. 13).

The histologic sections of the eyes of the rabbits killed at 9, 17, and 21 days showed a few scattered mononuclear and leukocytic cells. The edema and vasodilatation was less marked. Proliferation of the anterior epithelial and equatorial cells attempting to

Fig. 13 (Lyda and Lippincott). The findings of the left eye of a rabbit killed five days following the discission of the left lens. Small groups of mononuclear and leukocytic cells can be seen.

close the defect in the lens capsule had progressed remarkably so that much of the solid lens material was surrounded by these cells.

The relatively small amounts of cellular response seen in the sections of the eyes did not seem to correlate well with the intensity of the clinical response. Upon studying the sections it was felt that much of the clinical response was on the basis of a marked vasodilatation of the conjunctiva, iris, choroid, and ciliary body.

Comments

At the beginning of the study it was postulated that a nonspecific, generalized, hypersensitivity state was necessary to produce an endophthalmitis phacoanaphylactica in animals because so many of the patients seen with the disease had a history or some evidence of systemic allergies. However, in the first group of animals in which a systemic hypersensitivity state to ox serum was produced there was neither clinical nor histologic evidence of any sensitivity to lens material following discission of the lens. This finding led us to believe that under the conditions of the experiment rabbits required some factor other than a nonspecific hypersensitivity state to become systemically sensitized to lens material.

From the results of Burky and Woods[8] experiments it was decided that an adjuvant injected into the dispersed lens material might be the factor necessary for the development of a sensitivity to lens material.

The adjuvant chosen was staphylococcus toxin because this material had been used as an adjuvant by Burky and Woods[8] and because staphylococcus is one of the more common contaminants found in the conjunctival sac.

When staphylococcus toxin adjuvant was injected into the eyes of the ox-serum sensitized animals, there was neither a detectable reaction nor evidence of a phacoanaphylactic response. The changes noted were no greater than those found in the controls or in the first group of animals. Skin tests to lens antigen were negative, as well.

It was then decided that a systemic sensitivity to an adjuvant might be the necessary factor in producing the disease.

In the rabbits systemically sensitized to staphylococcus toxin and the toxin injected into the lens substance at the time of the discission of the right lenses, a marked inflammatory response developed. One week following this response skin tests to lens antigen were weakly positive.

We felt the inflammatory response produced was an ocular Arthus phenomenon to staphylococcus toxin. Lens material apparently participated in the reaction in such a way that a moderate systemic sensitivity to lens antigen developed. It would seem that the ocular Arthus phenomenon was necessary to produce a systemic lens sensitivity in rabbits, as negative results were obtained when the toxin was injected into nonspecifically sensitized rabbits.

When lens material was dispersed into the vitreous of the fellow eye in the lens-sensitized rabbits, there was a moderate inflammatory response not noted in any of the other groups.

This response was postulated as being an ocular Arthus phenomenon to lens substance in a lens sensitized animal. There seemed to be some support to the contention that the reaction was of an Arthus phenomenon type as the histologic findings in the two eyes were similar.

From the conditions of the experiment it would seem that the apparent operating mechanism in bilateral lens-induced endophthalmitis is an Arthus phenomenon type hypersensitivity reaction rather than one of an anaphylactic type hypersensitivity.

It was concluded that the factors necessary for the development of bilateral endophthalmitis phacoanaphylactica in rabbits are (1) a systemic sensitivity to an adjuvant that is later introduced into traumatized lens material so that an ocular Arthus phenomenon develops in the eye, followed by (2) the development of a systemic sensitivity to the released lens material and, finally, (3) damage to the lens of the fellow

eye with the liberation of adequate lens material so that an ocular Arthus phenomenon to lens material might occur.

Summary

1. Attempts to produce bilateral endophthalmitis phacoanaphylactica experimentally in rabbits are described and the results presented.

2. We found that in ox serum sensitized rabbits a simple discission of the lens or a discission followed by the injection of staphylococcus toxin into the eye produced a reaction no greater than that found in control animals. We also found that skin tests to lens antigen in these animals were negative.

3. Rabbits systemically sensitized to staphylococcus toxin and followed by a simple discission had an ocular reaction no greater than that found in the controls or in the eyes of the previous group.

4. The injection of staphylococcus toxin into the lenses of the staphylococcus sensitized animals at the time of the discission produced a marked inflammatory response that was considered to be an ocular Arthus phenomenon. Skin tests to lens antigen, although negative in all other groups, were weakly positive.

5. Discission of the lenses in the fellow eyes by the posterior route resulted in an inflammatory response that was not found in the other groups and was thought to be an ocular Arthus phenomenon to lens antigen.

6. From these findings it was concluded that for systemic sensitivity to lens antigen to develop in rabbits the animals must first be sensitized to an adjuvant and then the same material introduced into the lens of the traumatized eye. When systemic sensitivity to lens antigen is produced a relatively severe intraocular reaction occurs in the eye with the injured lens and when the lens of the fellow eye is posteriorly traumatized a phacoanaphylactic response seems to result.

7. It is postulated from the histologic findings that the response is an Arthus type hypersensitivity.

620 Cobb Building.

The lens antigen for this experiment was made available to us through the kindness of Dr. Jonas S. Friedenwald and Dr. Ronald Wood of the Wilmer Institute laboratories. We wish to acknowledge and thank Dr. R. L. Waugh, Jr., for his advice and counsel and to Dr. W. Topinka for his help with the animals.

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Discussion

Dr. Frederick H. Verhoeff (Boston, Massachusetts): This experimental work by Dr. Lyda and Dr. Lippincott needed to be done and has been done so well by them that it need not be repeated. Their conclusion, however, that they had produced in rabbits a bilateral phacoanaphylactic response is open to some doubt.

This is so because the inflammation was only

slightly greater in the left eyes than in the controls, and because it began to subside in four days, while there was still plenty of lens matter to cause its continuance. The resurgence of the inflammation in the right eye also contributes to the doubt.

It seems possible that, however produced, a moderately severe inflammation in one eye could so affect the other eye that a slight inflammatory reaction occurring in it about eight days later would be considerably enhanced. The reaction in the first eye might so disturb the vasomotor equilibrium of both eyes as not only to enhance a later reaction in the second, but also to cause a resurgence in the first eye.

In support of this possibility, I quote the following statement made by the essayists: "The relatively small amount of cellular response seen in the sections of the eyes did not seem to correlate well with the intensity of the clinical response. Upon studying the sections, it was felt that most of the clinical response was on the basis of a marked vasodilatation in the conjunctiva, iris, ciliary body, and choroid."

However, since there is no positive proof to the contrary, I assume the essayists' conclusion is correct.

Nevertheless, it seems to me that their experiments are of little significance as regards humans; and this is true also of all other animal experiments, including my own, which have to do with this question. Serious phacoanaphylactic endophthalmitis such as occurs in humans has never been experimentally produced. The reaction, as I have observed it in man, is relatively severe at the outset, and continues with increasing severity until the lens matter is absorbed, walled off, or removed by operation. The experimentally produced anaphylactic reaction is of slight severity, and subsides in a short time, while there is still an abundance of exposed lens matter.

I stated 33 years ago that injury to one lens does not sensitize the patient so that later injury to his other lens will produce an anaphylactic reaction. That this is true, except possibly under slightly unusual circumstances, is proven by the results of innumerable operations upon human eyes. Every time an ophthalmic surgeon performs an extracapsular extraction of cataract, he in effect unwittingly begins an experiment in answer to the question at issue, and he completes the experiment when he does a similar operation upon the other eye. Such operations show that, as a rule, the reaction in the second eye is substantially the same as in the first, and that a phacoanaphylactic reaction occurs in neither eye more often than can be explained by natural sensitivity to lens protein.

I feel sure that, if a laboratory animal is found which is naturally sensitive to lens protein, discission of one of his lenses will produce a phacoanaphylactic endophthalmitis worthy of the name. Should such an animal be found, an attempt should be made to breed a strain of lens-sensitive animals for experimental purposes.

Of course, the possibility exists that what I have termed phacoanaphylactic endophthalmitis is actually due to some other cause. But, in 33 years, no such cause has been discovered, and the evidence against it has become overwhelming, including especially the prompt and complete subsidence of the reaction on removal of the lens matter.

I will say here, if we are mistaken in assuming existence of this condition, the patient has reason to be grateful for the mistake, because his vision has been saved a good many times by removal of the lens matter.

The authors refer to the clinical cases of Courtney and to the case reported by Haik, Waugh, and Lyda. Time does not permit me to discuss these cases adequately; but it is my opinion that none of them proves that injury to the lens of one eye will sensitize a patient so that phacoanaphylactic endophthalmitis will occur in the other eye, even when its lens is injured.

The histologic findings in Dr. Haik's case exclude sympathetic uveitis, but do not prove phacoanaphylaxis. In fact, they strongly suggest that the reaction in the second eye, and possibly even in the first, was simply that of nongranulomatous uveitis due, as is usual, to unknown cause. If the reaction in the second eye had been an allergic reaction to to uninjured lens, I should have expected that the lens, after a year's time, would have been found encased in microphages and pus cells.

I wish to make clear that what I have said is in no sense a criticism of the experiments made by Dr. Lyda and Dr. Lippincott. I believe their experiments to be original, ingenious, and exceptionally well carried out; and I regard their results as important in showing that, in rabbits, injury to the lens of one eye, even when it produces systemic sensitivity to lens protein, does not lead to serious inflammation when, later, the lens of the other eye is injured.

DR. WILLIAM F. HUGHES, JR. (Chicago, Illinois): I cannot go back quite so far as 30 years but, about 15 years ago, we ran quite a series of skin tests in those individuals with extracapsular extractions and residual lens cortex, and we got quite a high correlation between the uveitis and the positive skin test to lens protein.

This was quite a crude product; and, in the last 10 years, using Parke-Davis material, we have found that very few of these cases show a positive test.

What sort of material was used in your cases, Dr. Lyda?

DR. WOOD LYDA (closing): I feel greatly honored to have Dr. Verhoeff discuss this paper and I am most appreciative to him for the helpful criticisms that he has offered and for his help in interpreting some of the perplexing results we found.

I would agree with Dr. Verhoeff that the reaction we produced in rabbits is not the same as the endophthalmitis phacoanaphylactica reaction seen in humans. In our experiments neither the severity, longevity, nor histologic picture conformed exactly to the clinical and histologic phenomenon as it is seen in humans.

However, it is our feeling that the bilateral re-

action we produced is an endophthalmitis phacoanaphylactica for rabbits.

It would be interesting to see if repeated, prolonged, and larger doses in rabbits or in monkeys might not more nearly simulate the human findings.

Our observation that the clinical inflammatory response seemed greater than the cellular response would warrant, was meant to be a comparison with the clinical histologic relationship usually seen in bacterial endophthalmitis. That we found a fairly marked vasodilatation of the intraocular vasculature seemed to us to point to the fact that the reaction was a hypersensitive rather than a bacterial one.

I believe that the conclusions that were drawn from the experiments were valid ones for rabbits. The point that we tried to emphasize was that under ordinary circumstances an extracapsular cataract extraction does not produce a phacoanaphylactic response. That for such a response to develop there must first be a systemic sensitivity to an antigen that is later introduced into the intraocular lens substance. Under these conditions a systemic sensitivity to lens material develops, as shown by weakly positive skin tests, and, if the fellow eye is traumatized or cataractous, an Arthus type anaphylactic response to lens antigen occurs.

In answer to the question concerning the longevity, or time interval, between the injection of the staphylococcus toxin into the lens of one eye and discission of the fellow eye: The staphylococcus toxin was injected into the first eye at the time of the discission in the first eye and discission of the fellow eye was done eight days after positive skin tests to lens antigen were found.

To Dr. Hughes' question: Dr. Ronald Wood of the Wilmer Institute was kind enough to supply us with the lens antigen used in these experiments.

On pilot studies before the experiment was carried out we tried a commercial product with negative results. In both the clinic and with the rabbits we obtained positive skin tests with the lens antigen from Wilmer Institute and at the same time in the same animal or patient would get negative results with the commercial product.

Symposium: Recent Trends in Diamox Research

* * *

THE EFFECTS OF THE CARBONIC ANHYDRASE INHIBITOR, ACETAZOLEAMIDE, ON THE COMPOSITION OF THE AQUEOUS HUMOR*

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I. INTRODUCTION

Systemic carbonic anhydrase inhibition by the administration of acetazoleamide and related compounds results in a partial suppression of secretion of aqueous humor.[1] These agents have proved most useful clinically in lowering intraocular pressure in glaucomatous eyes.[2-4] In addition, the ability to suppress partially the formation of the aqueous permits an evaluation of current concepts of the nature of the secretory process. Two of the constituents of the aqueous which are found in concentrations considerably in excess as compared with the plasma are ascorbic acid and bicarbonate. Both of these anions are considered to play key roles in the secretory process.[5,6] It is the purpose of this paper to report the alterations in the concentrations of these anions in the posterior and anterior aqueous humor induced by the carbonic anhydrase inhibitor, acetazoleamide. The changes found to date agree well with the predictions of Friedenwald-Kinsey theories, both as to the nature of the secretory product and the factors determining the composition of fluids in the anterior and posterior chambers of the eye. Furthermore, the alterations induced by acetazoleamide in constituents of the aqueous humor are consistent with estimations of suppression of aqueous secretion as measured by such independent methods as tonography.

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II. Methods

Albino rabbits weighing two to three kg. (Haskins Rabbitry, Saint Louis) were used throughout this study. Guinea pigs used were from the same source and weighed from 300 to 400 gm. Particular care was taken to avoid exciting animals. Results were found to be much more consistent when animals were kept in the room of the experiment in individual cages for at least 24 hours before tapping.

Blood was obtained by cardiac puncture, using a heparinized syringe. It was centrifuged at once in the cold. Blood for carbon dioxide analysis was introduced directly beneath U.S.P. paraffin oil. Posterior chamber fluid was drawn from the tetracaine anesthetized, proptosed eye into a calibrated pipet. It was found necessary to use a 26-gauge platinum needle embedded in the pipet to avoid metallic oxidation of ascorbic acid. Anterior chamber punctures were performed immediately after the removal of posterior chamber fluid, using a Kinsey 0.25 ml. calibrated pipet with a 24-gauge needle. The posterior chamber pipets were used for anterior chamber punctures in guinea pigs. The same pipets were used repeatedly, but cleaned and dried between uses by drawing hot dilute Sterox, distilled water, and absolute alcohol through them.

Nephrectomized animals had both kidneys removed under intravenous pentobarbital anesthesia through a single posterior incision 18 to 24 hours before use.

Acetazoleamide (Diamox*) was administered as the sodium salt to intact rabbits in doses of 100 mg./kg. intravenously, followed by 25 mg./kg. every 30 minutes for six hours. Nephrectomized animals received a single dose of 100 mg./kg. intravenously.

In order to "saturate" animals with ascorbic acid, nephrectomized animals were given 500 mg. of ascorbic acid at the time of nephrectomy. Intact animals received 50 mg. ascorbic acid subcutaneously every 30 minutes for six hours before the experiment began and continued for another six hours of acetazoleamide administration. This method of injection resulted in reasonably stable plasma ascorbic acid levels in the 15 to 30 mg. percent range.

Guinea pigs were given 10 mg./100 gm. of the sodium salt of acetazoleamide intraperitoneally as a single dose to nephrectomized animals and every 30 minutes to intact animals.

Total carbon dioxide content was determined by the method of Van Slyke. Bicarbonate values were obtained by correcting for carbon dioxide, assuming the pH values of normal rabbit plasma (7.40) and aqueous (7.55) described by Kinsey.* Since this correction is very small, alterations in aqueous pH values induced by acetazoleamide change these data very little. All values are expressed in millimols per liter of water.

Ascorbic acid in four-percent metaphosphoric acid was titrated immediately with dichlorphenolindophenol, using a 0.1 ml. Gilmont ultramicroburet.

All experiments were arranged so that an initial blood and the fluids from one eye were drawn; the animals were then subjected to carbonic anhydrase inhibition for a given time interval; then the second samples of blood and fluids from the other eye were obtained. Every effort was made to use the same time intervals for centrifugation, running the determinations, and so forth, on the samples before and after acetazoleamide.

III. Results

A. Bicarbonate

Steady-state concentrations of bicarbonate in the posterior and anterior chambers were found to be in excess of plasma concentration by an average of 68 percent and 36 percent respectively. In each of the 30 animals tested, there was a considerable fall in both posterior chamber and anterior chamber concentrations of bicarbonate following six hours of carbonic anhydrase inhibition, but

* Supplied through the courtesy of Dr. James D. Gallagher, Clinical Research Section, Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

very little change in plasma levels (table 1). The average fall in posterior chamber bicarbonate was 8.4 mM/L, or from an excess over plasma of 68 percent down to an excess of 38 percent. The anterior chamber bicarbonate concentration fell an average of 7.2 mM/L, or from an excess over plasma of 68 percent down to an excess of only 10 percent. These changes proved statistically significant. There was no significant difference between nephrectomized and intact animals except for a tendency for the plasma bicarbonate to fall slightly in intact animals. This is probably a result of renal diuresis of bicarbonate under the influence of acetazoleamide.

Since these are essentially steady state data before and after carbonic anhydrase inhibition, one may apply the simplified formula of Kinsey and Friedenwald:[7]

$$\frac{K_F}{K_D} = \frac{C_{AC} - C_{PI}}{C_{PC} - C_{AC}}$$

where K_F is the coefficient of transfer by flow into and out of the anterior chamber;

K_D = the coefficient of transfer by diffusion between blood and anterior chamber;

C_{AC} = steady state concentration in the anterior chamber;

C_{PC} = steady state concentration in the posterior chamber;

C_{PI} = steady state concentration in the plasma.

The comparison of steady state data be-

TABLE 1

BICARBONATE RABBIT AQUEOUS (MM/L WATER)

	Before Acetazoleamide					Six Hours After Acetazoleamide					
	C_{PI}	C_{PC}	C_{PC}/C_{PI}	C_{AC}	C_{AC}/C_{PI}	C_{PI}	C_{PC}	C_{PC}/C_{PI}	C_{AC}	C_{AC}/C_{PI}	$\Delta F\%$
	22.8	—	—	33.7	1.48	21.0	—	—	22.9	1.09	—
	24.9	—	—	35.2	1.41	24.0	—	—	27.4	1.14	—
	28.7	—	—	40.0	1.39	25.2	—	—	27.2	1.08	—
	32.5	—	—	39.3	1.21	32.5	—	—	33.3	1.03	—
	22.6	—	—	31.5	1.40	20.5	—	—	23.1	1.12	—
	18.8	—	—	29.3	1.56	20.5	—	—	23.2	1.13	—
	28.2	42.3	1.50	33.2	1.18	21.7	30.5	1.40	23.2	1.07	−64
	23.1	37.4	1.62	30.0	1.30	23.1	33.5	1.45	25.0	1.08	−76
	24.8	43.0	1.73	32.8	1.32	23.1	30.8	1.33	25.1	1.09	−56
	21.8	34.9	1.60	27.5	1.26	20.3	26.2	1.29	22.0	1.08	−48
	24.8	44.8	1.81	33.5	1.35	20.0	30.8	1.54	22.7	1.14	−57
	25.4	37.3	1.47	32.5	1.28	19.9	31.5	1.58	22.9	1.15	−75
	25.7	43.7	1.70	34.7	1.35	26.2	35.4	1.35	28.8	1.10	−61
	21.5	40.4	1.88	32.0	1.49	22.0	28.6	1.30	24.1	1.09	−63
	24.3	42.6	1.75	34.2	1.41	21.9	30.6	1.40	24.5	1.12	−64
	*20.6	31.3	1.52	25.9	1.26	20.3	29.2	1.44	22.6	1.11	−65
	*19.9	31.9	1.60	25.4	1.28	18.3	26.8	1.46	20.8	1.14	−51
	*24.0	42.7	1.78	32.9	1.37	26.1	36.7	1.41	28.0	1.07	−76
	*21.0	40.3	1.92	30.1	1.43	20.2	28.6	1.42	23.0	1.14	−44
	*20.3	36.9	1.81	28.4	1.40	20.9	28.7	1.32	23.0	1.10	−61
	*23.2	40.8	1.76	32.0	1.38	22.6	32.4	1.43	25.3	1.12	−62
	*19.3	35.7	1.85	29.0	1.50	18.4	28.0	1.52	21.2	1.15	−71
	*24.8	42.2	1.70	34.0	1.37	23.8	33.0	1.39	25.5	1.07	−80
	*25.4	46.7	1.84	35.2	1.38	25.0	32.9	1.32	26.1	1.04	−81
	*21.8	35.6	1.63	30.0	1.37	22.7	28.6	1.26	24.0	1.06	−81
	*19.8	36.5	1.83	28.6	1.44	20.8	29.2	1.40	22.7	1.09	−74
	*28.2	38.8	1.37	35.2	1.25	27.7	32.0	1.16	29.7	1.17	−55
	*25.6	39.1	1.53	31.9	1.24	26.0	33.4	1.29	28.2	1.08	−52
	*28.4	42.6	1.50	36.8	1.30	27.4	32.8	1.20	29.9	1.09	−40
	*25.9	42.9	1.65	35.5	1.37	25.3	37.5	1.48	29.0	1.14	−66
Mean	23.9	39.6	1.68	32.3	1.36	22.9	31.2	1.38	25.1	1.10	−64%
S.D.	±3.1	±4.0	±0.14	±3.5	±0.09	±3.1	±2.9	±0.14	±2.9	±0.03	+11%

* Nephrectomized rabbit.

C_{PC} = posterior chamber concentration; C_{AC} = anterior chamber concentration; C_{PI} = plasma concentration $t_{PC/PI} = 8.1$; $t_{AC/PI} = 14.8$; $t_{PC} = 8.4$; $t_{AC} = 9$; $\Delta F\%$ = per cent change aqueous flow.

fore and after carbonic anhydrase inhibition thus provides us with a measure of change in the ratio $\frac{K_F}{K_D}$. If K_D may be assumed to remain unchanged following acetazoleamide administration, then these data may be used to estimate relative changes in K_F, the rate of aqueous flow. From measurements of the turnover of test substances in the ocular fluids, there is evidence that K_D is not significantly altered by carbonic anhydrase inhibition.[8] The agreement among values reported in this paper for change in flow induced by Diamox and especially the close similarity to results obtained by such completely independent methods as tonography appear to justify the assumption of an insignificant change in K_D. When such estimations were applied to the bicarbonate data, they were found to be consistent with an average of 64 percent decrease in aqueous flow (ΔF percent in table 1).

The carbon dioxide content of the anterior chamber aqueous of the control eyes of 18 guinea pigs was found to average 36.8 mM/L or 70 percent in excess of plasma. Following six hours of carbonic anhydrase inhibition, this fell to an average of 26.5 mM/L or 19 percent excess over plasma, an average statistically significant fall of 10.3 mM/L water (table 2). Thus, the bicarbonate excess in the aqueous humor and its reduction following acetazoleamide administration are not confined to rabbit eyes.

In short-term experiments (approximately 25 minutes) on intact rabbits, the results of a single administration of 100 mg./kg. acetazoleamide intravenously are presented in Table 3. At this short time interval there was no significant change induced in anterior chamber bicarbonate concentration (an average fall of 1.2 mM/L). However, the posterior chamber bicarbonate concentration had fallen significantly in 25 minutes (an average fall of 6.2 mM/L) and had almost reached its new steady state level. These findings are in good agreement with current concepts of the more rapid rate of turnover of substances in the smaller posterior chamber as compared with the larger anterior chamber. The rapid decrease in posterior chamber bicarbonate correlates well with the time course of the intraocular pressure fall.

TABLE 2

CO_2 CONTENT GUINEA PIG AQUEOUS (mM/L WATER)

	Before Acetazoleamide			Six Hours After Acetazoleamide		
	C_{PI}	C_{AC}	C_{AC}/C_{PI}	C_{PI}	C_{AC}	C_{AC}/C_{PI}
	19.5	37.1	1.90	21.1	26.4	1.25
	17.9	33.5	1.87	22.0	25.4	1.15
	18.6	37.3	2.00	23.7	27.1	1.14
	18.0	34.4	1.91	25.6	27.7	1.08
	20.0	37.0	1.85	20.8	24.6	1.18
	20.2	32.2	1.60	22.7	26.5	1.17
	20.6	34.2	1.66	19.4	23.1	1.19
	22.5	36.4	1.62	24.1	28.5	1.18
	*22.8	37.5	1.65	21.6	26.3	1.22
	*26.8	40.1	1.50	24.8	28.1	1.13
	*21.4	35.7	1.67	23.6	28.0	1.19
	*18.4	34.6	1.88	19.1	24.2	1.27
	*22.8	40.7	1.79	21.3	26.1	1.22
	*18.1	35.2	1.95	19.4	26.3	1.35
	*21.6	39.2	1.82	20.5	23.2	1.13
	*20.9	36.1	1.73	22.1	26.7	1.21
	*26.1	38.5	1.48	26.4	29.2	1.11
	*25.8	41.8	1.62	24.2	28.8	1.19
Mean	21.2	36.8	1.70	22.4	26.5	1.19
S.D.			±0.16			±0.06

* Nephrectomized.

C_{AC} = anterior chamber concentration; C_{PI} = plasma concentration; $t_{AC/PI} = 12.8$.

B. Ascorbic acid

The concentration of ascorbic acid in the posterior chamber rose after six hours of acetazoleamide by approximately 50 percent from an average value of 28.9 mg. percent (1.64 mM/L) to 43.2 mg. percent (2.46 mM/L). There was also an increase in ascorbic acid in the anterior chamber, but only by about 14 percent from the normal average value of 22.8 mg. percent (1.30 mM/L) to 25.9 mg. percent (1.47 mM/L) (table 4). Similar rises took place in the animal with elevated plasma ascorbic acid (table 5). Here it will be noted that the saturation level of ascorbic acid for the posterior chamber averaged 76 mg. percent (4.32 mM/L) and increased to 113 mg. percent (6.44 mM/L), a value significantly higher than has been encountered in the rabbit not subjected to acetazoleamide even at plasma levels of several hundred mg. percent.[9] This was also approximately a 50 percent rise in posterior chamber concentration of ascorbic acid.

TABLE 3

BICARBONATE RABBIT AQUEOUS (MM/L WATER)

	Before Acetazoleamide					After Acetazoleamide					Tme (min.)
	C_{PI}	C_{PC}	C_{PC}/C_{PI}	C_{AC}	C_{AC}/C_{PI}	C_{PI}	C_{PC}	C_{PC}/C_{PI}	C_{AC}	C_{AC}/C_{PI}	
	20.9	37.8	1.81	29.6	1.42	22.0	32.7	1.48	29.5	1.34	22
	19.3	35.6	1.85	27.6	1.43	22.4	29.8	1.33	27.1	1.21	25
	23.0	38.2	1.66	30.2	1.31	22.0	33.2	1.51	29.8	1.35	24
	19.3	37.3	1.93	28.2	1.46	22.6	31.6	1.40	28.0	1.24	25
	18.2	32.6	1.79	26.8	1.47	18.5	27.8	1.50	24.6	1.33	25
	26.6	41.4	1.56	30.2	1.14	27.6	35.4	1.28	29.4	1.07	25
	26.2	41.7	1.59	33.2	1.27	23.2	34.1	1.47	31.4	1.35	25
	24.3	45.8	1.89	35.2	1.45	21.9	36.7	1.67	33.2	1.51	25
	21.5	40.4	1.88	32.0	1.49	22.0	32.6	1.48	29.3	1.33	20
	19.2	35.2	1.84	28.8	1.50	19.7	29.0	1.47	27.2	1.38	25
Mean	21.9	38.5	1.78	30.2	1.39	22.2	32.3	1.45	29.0	1.31	
S.D.	±3.1	±3.4	±0.13	±2.4	±0.11	±2.3	±2.5	±0.14	±2.2	±0.11	

C_{PC} = concentration in posterior chamber aqueous
C_{AC} = concentration in anterior chamber aqueous
C_{PI} = concentration in plasma

$t_{PC/PI} = 5$
$t_{AC/PI} = 1.5$
$t_{PC} = 4.1$
$t_{AC} = 1.1$

There was a lesser increase in the anterior chamber ascorbic concentration from an average of 66 mg. percent (3.75 mM/L) to 81.8 mg. percent (4.65 mM/L), or about 24 percent. The change in aqueous flow, as calculated by the alteration in $\frac{K_F}{K_D}$ is presented in Tables 4 and 5 assuming no change in K_D. For the 28 animals at normal ascorbic-acid levels, the inhibition of flow was an average of 60 percent. Those with elevated plasma ascorbic acid levels had an average inhibition of flow of 51 percent. The difference between these two groups of animals is significant only at $t = 2.3$. This tendency of ascorbic acid to reverse partially the inhibition of flow induced by carbonic anhydrase inhibition is of considerable interest. It correlates well with the partial reversal of the effects of acetazoleamide on intraocular pressure.[10] However, much more data are needed before one can more than speculate about the effects of ascorbic acid on aqueous flow. Tonographic and other methods are now being applied to this problem.

TABLE 4

ASCORBIC ACID RABBIT AQUEOUS (MG. %)

	Before Acetazoleamide		Six Hours After Acetazoleamide		
	C_{PC}	C_{AC}	C_{PC}	C_{AC}	ΔF%
	24.8	18.5	40.5	23.6	−53
	31.6	25.4	45.7	27.4	−63
	32.4	22.5	48.8	24.3	−56
	22.2	17.2	35.6	19.6	−64
	28.3	21.2	40.7	23.6	−54
	31.2	24.6	47.2	30.0	−53
	34.3	26.8	42.4	29.1	−39
	20.6	14.2	27.8	16.6	−34
	41.2	35.4	60.0	41.0	−65
	35.6	27.0	50.6	30.6	−51
	*44.0	36.9	61.4	38.0	−69
	*39.3	32.5	66.5	36.5	−74
	*18.6	13.9	29.8	15.2	−65
	*23.8	17.7	34.0	19.0	−56
	*23.6	16.8	38.2	19.1	−59
	*27.5	18.6	38.5	17.8	−59
	*35.4	26.3	56.9	31.4	−56
	*19.7	15.1	28.3	13.7	−71
	*26.3	22.0	35.8	21.5	−71
	*36.7	31.0	48.5	35.5	−50
	*39.5	30.8	56.5	36.0	−50
	*26.7	23.4	39.4	29.6	−56
	*20.0	13.8	29.8	15.0	−54
	*27.7	24.6	41.8	30.2	−67
	*29.9	27.0	44.5	30.3	−77
	*26.8	24.4	50.6	33.0	−81
	*24.2	18.5	38.6	22.0	−59
	*17.0	12.6	29.8	14.4	−67
Mean (mg. %)	28.9	22.8	43.2	25.9	−60%
					S.D. ±10%
Mean (mM/L)	1.64	1.30	2.46	1.47	

ΔF% = per cent change in aqueous flow assuming plasma ascorbic is negligibly small and no change in diffusion constant.
* = nephrectomized rabbit
C_{PC} = posterior chamber concentration
C_{AC} = anterior chamber concentration.

III. Discussion

The large excess of bicarbonate in the posterior chamber of the eye suggested a role of carbonic anhydrase in the formation of this secretion. The finding of a reduction in this bicarbonate excess by carbonic an-

TABLE 5

ASCORBIC ACID RABBIT AQUEOUS (MG. %)
(elevated plasma ascorbic)

	Before Acetazoleamide			After Acetazoleamide			
	C_{PC}	C_{AC}	C_{PI}	C_{PC}	C_{AC}	C_{PI}	ΔF%
	76.5	63.0	21.5	112.5	82.2	21.2	−34
	75.8	63.8	20.6	112.0	78.2	16.4	−46
	73.1	60.0	13.5	115.0	81.4	15.6	−44
	77.4	62.1	17.9	111.0	80.1	20.1	−33
	76.0	66.2	25.6	118.0	79.5	29.8	−49
	73.9	65.4	22.5	114.0	88.3	21.9	−48
	72.2	64.5	28.8	120.0	86.0	30.0	−64
	*74.2	64.2	22.6	116.0	78.7	17.6	−61
	*79.5	66.5	28.5	120.0	80.0	26.2	−54
	*82.5	72.5	29.2	120.0	78.5	22.0	−69
	*71.0	60.6	18.3	103.0	70.5	10.5	−56
	*79.0	74.0	28.6	113.0	84.0	23.3	−77
	*69.5	60.0	22.0	110.0	86.2	19.1	−30
	*81.6	72.0	32.2	105.0	80.2	28.2	−48
	*80.0	74.5	32.4	109.0	92.5	29.4	−50
Mean (mg. %)	76.1	66.0	24.3	113.2	81.8	22.8	−51% S.D. ±13%
Mean (mM/L)	4.32	3.75	1.38	6.44	4.65	1.30	

* Nephrectomized rabbit.
C_{PC} = posterior chamber concentration
C_{AC} = anterior chamber concentration
C_{PI} = plasma concentration
ΔF% = per cent change in aqueous flow.

hydrase inhibition strongly suggests a relationship between carbonic anhydrase and the bicarbonate excess found in the eye. It is of some interest that the decrease of bicarbonate excess in the posterior chamber is of the same order of magnitude as estimates of decreased secretion induced by acetazoleamide.

An even greater relative fall in bicarbonate concentration is measured in the anterior chamber fluid. Here, in addition to the lower concentration of bicarbonate entering from the posterior chamber, the partial stagnation of aqueous provides a longer time interval than normal for diffusion of bicarbonate out of the anterior chamber and a tendency toward equilibration with plasma bicarbonate levels.

The failure of Green et al.[11] to find significant changes in anterior chamber bicarbonate at 30-minute intervals after acetazoleamide administration can be verified (see table 3). This is easily explained, however, when one observes that the posterior chamber concentration of bicarbonate has changed almost maximally at 25 minutes, but that the anterior chamber effect takes longer than this period of time to reach a magnitude that exceeds the experimental error of measurement.

Unfortunately Green and co-workers[11] confined their observations to the *anterior chamber* and *at short time intervals*, thus missing the profound rapid changes in the posterior chamber fluids and slower alterations in the anterior chamber bicarbonate concentrations. It is clear, therefore, that there is little justification for their conclusions that carbonic anhydrase activity is not necessary for the elaboration or maintenance of bicarbonate ions in the aqueous humor of rabbit eyes. On the contrary, *there is every indication that aqueous bicarbonate concentrations as well as the rate of secretion of aqueous humor are dependent upon carbonic anhydrase activity.*

The rise in posterior chamber ascorbic acid remains unexplained. There are at least three possible explanations for this phenomenon:

1. All or part of the ascorbic acid might be secreted by a related or independent mechanism that is less dependent on carbonic anhydrase than the secretion of bicarbonate and inflow of water. Since the inflow of water would thus be decreased more effectively than the secretion of ascorbic acid, the concentration of ascorbic acid would rise. The amount of rise could be as much as 150 percent if ascorbic acid secretion were not inhibited at all and water inflow was depressed 60 percent. The actual rise found is only 50 percent however. This would indicate either a lesser effect on this ascorbic secretory mechanism or a separate source of ascorbic acid that is not dependent upon carbonic anhydrase. At any rate, it is clear that the entrance of ascorbic acid into the eye is partially inhibited by acetazoleamide in spite of the fact that its concentration rises.

1a. A special case of other sources of ascorbic acid could conceivably be intraocular formation of a part of the ascorbic acid of the aqueous by such structures as the lens. This would result in a rise in aqueous ascorbic concentration due to the partial stagnation. However, this would not be consistent with the finding of a 50-percent rise in the posterior chamber concentration of ascorbic acid at elevated plasma level (that is, a 37 mg. percent rise saturated versus 14 mg. percent at normal plasma level). Furthermore, the evidence presented by Friedenwald[9] makes it quite unlikely that ascorbic-acid formation occurs in the eye.

2. A rise in plasma ascorbic acid might account for the change in ascorbic acid concentration in the eye. However, this cannot explain the findings in the rabbits whose plasma ascorbic acid was maintained at high levels (table 5). Here the posterior chamber concentration of ascorbic acid reached a level higher than saturation for the normal rabbit eye. Since the percent rise after acetazoleamide in these animals saturated with ascorbic acid is almost precisely the same as those at low plasma levels, the factor of a possible rise in plasma ascorbic acid concentration seems extremely unlikely.

3. Ascorbic acid might enter the posterior chamber fluids in greater amounts during carbonic anhydrase inhibition due to its function in the secretory mechanism and the alteration in that role in the partially suppressed activity associated with carbonic anhydrase inhibition. This concept is consistent with the Friedenwald hypothesis as to the possible mechanism of action of acetazoleamide on the secretion of aqueous.[10]

Efforts are now in progress to attempt to elucidate the relative roles of these different possibilities in the production of a rise in ascorbic acid in ocular fluids following carbonic anhydrase administration.

The close agreement between the average estimates of inhibition of flow as measured by bicarbonate steady state (64 percent) and ascorbic acid steady state (60 percent) data is most stimulating. These data are summarized in Table 6. Even more exciting is the comparison of these estimates with those obtained by tonography. Thus in a series of 50 rabbit eyes, repeated tonography before and after systemic carbonic anhydrase inhibition indicated an average

TABLE 6

SUMMARY OF AVERAGE STEADY-STATE DATA (MM/L)

Substance	No. Rabbits	Before Acetazoleamide			Six Hours After Acetazoleamide			ΔF
		C_{PI}	C_{PC}	C_{AC}	C_{PI}	C_{PC}	C_{AC}	
Bicarbonate	24	23.9	39.6	32.3	22.9	31.2	25.1	−64%
Ascorbic Acid	28	—	1.64	1.30	—	2.46	1.47	−60%
Saturated Ascorbic	15	1.38	4.32	3.75	1.30	6.44	4.65	−51%

C_{PI} = concentration in plasma.
C_{PC} = concentration in posterior chamber aqueous.
C_{AC} = concentration in anterior chamber aqueous.
ΔF = change in aqueous flow.

suppression of secretion of 63 percent.[12] Comparison of tonograms and steady state bicarbonate changes in the same animal revealed an even closer agreement of the two methods. Furthermore, repeated tonograms on 59 human eyes before and after acetazoleamide revealed an average decrease of aqueous flow of 61 percent.[13] The agreement of average data obtained by such different methods tends to establish on firm ground that carbonic anhydrase inhibition results in an average of some 60 to 65 percent inhibition of aqueous flow. It also tends to validate present concepts of aqueous humor dynamics and methods of measurement of aqueous flow.

IV. Summary

1. The systemic administration of acetazoleamide, a carbonic anhydrase inhibitor, to 30 rabbits resulted in a decrease in the steady state bicarbonate concentration in the posterior chamber aqueous from 15.7 mM/L (68 percent ± 14 percent) in excess of plasma levels down to 8.3 mM/L (38 percent ± 14 percent) above plasma, and in the anterior chamber from 8.4 mM/L (36 percent ± 9 percent) to 2.2 mM/L (10 percent ± 3 percent) excess compared to plasma.

2. The fall in rabbit posterior chamber bicarbonate occurred rapidly and correlated well with the decrease in aqueous secretion and lowering of intraocular pressure. The alteration in the anterior chamber level was considerably slower.

3. The anterior chamber carbon-dioxide content in the eyes of 18 guinea pigs was 70 percent ± 16 percent in excess of plasma level and fell after acetazoleamide to 19 percent ± 6 percent above the plasma concentration.

4. The average steady state concentration of ascorbic acid was increased by approximately 50 percent in the posterior and 14 percent in the anterior chamber following acetazoleamide administration to 28 rabbits.

5. The changes in composition of rabbit aqueous humor are consistent with an average suppression of aqueous flow of 64 percent ± 11 percent (bicarbonate) and 60 percent ± 10 percent (ascorbic acid) as a result of carbonic anhydrase inhibition. These estimates are in excellent agreement with tonographic data.

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EFFECTS OF LOCALLY ADMINISTERED DIAMOX*

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The hypotensive action of Diamox upon glaucomatous eyes has been shown to be related to the route of administration. Local administration of the drug has been shown to have no effect upon the intraocular pressure of human eyes.

The experimental observations of Grant and Trotter,[1] Foss,[2] Harris,[3] and Green, Leopold, and co-workers[4] clearly indicate that the drug exerts no appreciable effect upon the intraocular pressure of normal rabbit eyes when given subconjunctivally, intracamerally, iontophoretically, or by the instillation of drops.

It was, therefore, considered important, relative to an understanding of the mechanism of action of Diamox, to study the effects of locally administered Diamox upon the chemistry of the intraocular fluids and of the anterior uvea of the normal rabbit eye.

In a recent publication from the Wills laboratory[5] it was shown, by a spectrophotometric method, that measurable quantities of the drug were present in the anterior chamber aqueous humor of the rabbit eye up to 90 minutes after subconjunctival injection of 10 mg. of Diamox. By enzyme-inhibition studies, the drug was also detected in the anterior uveal tissues.

Because of the prominent role that the enzyme carbonic anhydrase may play in the elaboration of HCO_3^- in the aqueous humor, and in the regulation of the intraocular pressure, the effect of the subconjunctival injection of Diamox upon the activity of the enzyme in the anterior uvea was determined. As previously reported,[4] the enzyme activity of extracts of the anterior uvea was completely inhibited by the subconjunctival injection of Diamox. Since Diamox is a potent carbonic anhydrase inhibitor, being active in relatively small amounts, it was important to establish whether the experimentally measured inhibition reflected the actual in vivo state of affairs, or was due to contamination of the tissue extract by adsorption of small amounts of the drug in surface contact with the tissue.

Evidence indicating that a true in vivo inhibition of the enzyme occurred was obtained by the following experiment. The ciliary body-iris tissue of an untreated normal rabbit eye was removed and immersed in two successive saline solutions that had been previously used for the wash-by-immersion of a tissue whose carbonic anhydrase activity was completely inhibited as a result of the subconjunctival injection of Diamox. After immersion in fresh saline, the extract of the tissue was prepared and found to possess the original level of carbonic anhydrase activity previously found for normal tissues.

Since the ciliary body is considered as the site of the secretory mechanism, it was necessary to determine whether the enzyme activity in the ciliary body was inhibited by the subconjunctival injection of Diamox. For these experiments the ciliary body was separated from the iris by the procedure of Friedenwald and co-workers.[6] Results of the assay of the enzyme activity in the extracts of the separated tissues indicate that the ciliary body possessed 60 percent and the iris 40 percent of the total activity of the anterior uvea. And in separate experiments it was found that the subconjunctival administration of 10 mg. of Diamox completely inhibited the activity in each tissue.[4]

Chemical analyses of the aqueous humor as previously reported,[7] showed that the sub-

* From the Wills Eye Hospital, Department of Research. Most of the data to be presented have been published. This presentation is a summary report of the results.

TABLE 1

EFFECT OF SUBCONJUNCTIVAL INJECTION OF 10 MG. DIAMOX IN 0.2 ML. H_2O UPON THE BICARBONATE-ION CONCENTRATION OF THE AQUEOUS HUMOR IN THE POSTERIOR CHAMBER OF THE RABBIT EYE

(Values are expressed as mM/L.)

	O.S. (0 min.)	O.D. (30 min.)	O.S. (0 min.)	O.D. (60 min.)
	37.4	36.6	46.8	47.4
	35.7	45.2	36.9	39.3
	37.4	41.6	40.7	40.6
	36.1	46.5	44.9	45.8
	43.6	44.1	38.0	34.2
	39.1	35.8	41.7	43.6
	37.7	37.4	40.8	36.8
	35.4	32.8	42.9	35.9
	37.8	32.4	42.9	37.4
	41.5	32.3	42.9	38.8
AVERAGE	38.2	38.5	41.9	40.0
Standard Deviation	2.5	5.2	2.8	4.1

conjunctival injection of from five to 20 mg. of Diamox did not significantly lower the bicarbonate ion concentration in the anterior chamber after 30, 60, and 180 minutes. More recently the aqueous humor from the posterior chamber has been analyzed. The results in Table 1 show that the subconjunctival injection of 10 mg. Diamox had no significant effect upon the bicarbonate ion concentration in 30 and 60 minutes.*

During the course of this investigation it was observed that occasionally the aqueous humor that was removed from an eye that had been injected subconjunctivally with Diamox became a gel on standing. This phenomenon of gelation was frequently encountered in our studies with plasmoid aqueous humor and suggested that the subconjunctival injection of Diamox increased the permeability of the blood-aqueous barriers.

That this was the case was confirmed by protein analyses of the aqueous humor and by determining the fluorescein appearance time in the anterior chamber. The results show that the subconjunctival injection of 10 to 20 mg. of Diamox, pH 8.8 to 9.0, caused in some animals a marked increase in the protein concentration of the aqueous humor in 30 to 60 minutes; the time of appearance of fluorescein in the anterior chamber was decreased by about 50 percent.[4]

Since the injection of isotonic saline at the same pH had no appreciable effect upon the protein concentration of the anterior chamber, it is reasonable to conclude that the injection of Diamox under the experimental conditions effectively increased the permeability of the blood-aqueous barriers. This phenomenon may be a factor contributing to the absence of any hypotensive activity of Diamox when used subconjunctivally.

CONCLUSION

The failure of locally administered Diamox to lower intraocular pressure of normal rabbit eyes may be taken as presumptive evidence that Diamox under these conditions did not inhibit the carbonic anhydrase of the ciliary body. The evidence presented here, however, indicates that the carbonic anhydrase was inhibited. The possibility that this inhibition occurred after death of the animal rather than in vivo has not been excluded entirely.

The fact that bicarbonate levels in the posterior and anterior chambers fail to change after local Diamox also may be taken as presumptive evidence that the carbonic anhydrase was not inhibited; or it may indicate that inhibition of the enzyme has no apparent effect upon the $[HCO_3^-]$ in the aqueous humor of the normal rabbit eye.

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* For analysis 20 to 30 µl. of aqueous humor was removed from the posterior chamber by means of a microburet with a sealed in platinum 26-gauge needle. The pipettes were made by James Graham, University of Pennsylvania. Each pipette was calibrated against water for volume delivery and against standard bicarbonate solution by the Warburg technique. The probable error of the method to be expected is within five percent; the extreme error among the observations was less than 10 percent.

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CURRENT STUDIES ON ACETAZOLEAMIDE (DIAMOX) AND AQUEOUS HUMOR FLOW*

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It is my function in this symposium to attempt to sum up the present state of our knowledge of the influence of Diamox on intraocular pressure and aqueous humor flow. The developments in this field have been so rapid that my account must, in some respects, be incomplete.

Those of you who attended the session on tonography and aqueous humor flow of the International Congress of Ophthalmology in New York only nine months ago will remember Dr. Kinsey and Dr. Palm's[1] important contribution in which, by studying the turnover of test substances in both the anterior and posterior chambers they were able to obtain a new and more reliable estimate of the rate of flow of the intraocular fluid in rabbits. Two hundred experiments were required and an estimate with an uncertainty of approximately 20 percent was obtained.

Once a reliable method of measuring the rate of flow had been achieved the next question was the application of such a method to the study of the effect of a whole series of experimental variables. Those of us who contemplated this problem were appalled at its magnitude. Were we going to have to use several hundreds of rabbits and many months of work to find the effect of each of the many variables that needed to be tested? Efforts were made to find short-cuts and several of these have been partially successful.

At the Wilmer Institute, Dr. Kornblüth and Dr. Linnér[2] have investigated the applicability of tonography to rabbits. It develops that in some rabbits weighing two to three kg. the ocular rigidity and corneal curvature are sufficiently near to those of man to permit the use of human calibration tables for readings on rabbits. This good agreement with the human calibration is not universal among rabbits and the application of human calibration tables to rabbits must be made with great caution. Estimates on the rate of aqueous flow made by tonographic procedure on normal rabbits agree remarkably well with Kinsey's estimates of the rate of flow based on chemical turnover rates. Comparing estimates on the same

* From the Wilmer Ophthalmological Institute of the Johns Hopkins Hospital. Work at the Wilmer Institute in this field has been supported in part by the Kellogg Foundation, by a graduate training grant from the National Institutes of Health, and by a gift from the American Cyanamid Corporation.

rabbit before and after a given treatment or comparing the two eyes of the same rabbit one can greatly reduce the influence of residual systematic errors and enhance the sensitivity of the method.

Dr. Linnér and I[3] have also developed the use of the appearance time of fluorescein as an index of the rate of aqueous flow. For these animals in which fluorescein enters the aqueous chiefly via the posterior chamber, the time, after an intravenous injection, between the arrival of fluorescein in the intraocular vessels and its arrival in the pupil may be taken as a measure of the rate of aqueous flow. Variation in the appearance time from animal to animal is considerable, but reproducibility of the measurement on the same eye is fair if the animals are not excited and struggling. Experiments comparing the two eyes of the same animal or the same eye before and after a given treatment yield estimates of the experimentally induced changes in aqueous flow even if the absolute value of the flow rate is unknown. When the flow of aqueous is extremely slow, the fluorescein appearance time tends to overestimate the rate of flow since, even with a normal blood aqueous barrier, some diffusion through the iris occurs and may be confused with transport of the dye through the pupil. Thus Becker finds the flow inhibition by Diamox to be underestimated with the fluorescein method as compared with tonography.

Dr. Becker[4] has studied the concentration ratios of ascorbate and of bicarbonate in anterior chamber, posterior chamber, and plasma before and after these have been shifted from one steady-state level to another by some experimental procedure. If it is assumed that the experimental procedure has not changed diffusion rates across the blood anterior chamber barrier, then a change in the steady state ratios can be used to estimate changes in the rate of aqueous flow. The intercomparison of all these various estimates of aqueous flow together with dynamic studies on the Kinsey model shows remarkably good agreement between the various methods in certain types of experiments. Each of these procedures has its own special limitations. Tonography cannot be applied, at least not without elaborate special corrections to experiments which alter ocular rigidity or episcleral venous pressure. Fluorescein appearance time and steady state concentration ratios are inapplicable to experiments in which the blood aqueous barrier is damaged.

The application of all these methods to experimental animals and of tonography and fluorescein appearance time to humans has proven conclusively that Diamox diminishes the rate of inflow of the aqueous without directly affecting the resistance to outflow.[5] This is in sharp contradistinction to the conventional anti-glaucomatous agents, the miotics which lower intraocular pressure by increasing facility of outflow without directly influencing the rate of inflow.

Considering the difficulties and uncertainties that afflicted our science less than a year ago this is indeed solid achievement. But every question answered brings us to a new question. What are the conditions under which this lowering of intraocular pressure by Diamox takes place? An approach to this question can be constructed through consideration of the fact that Diamox is an inhibitor of the enzyme carbonic anhydrase. We may ask, assuming that carbonic anhydrase facilitates the secretory flow of intraocular fluid, what are the conditions that presentation of the inhibitor of this enzyme will lower intraocular pressure? I should like to discuss this question under a number of headings.

1. Surplus enzyme

If an enzyme is essential to a certain physiologic function, complete inhibition of the enzyme must be associated with inhibition of the function—but the quantitative relations are not simple and are influenced for instance by the surplus of enzyme present locally. Many physiologic functions are pro-

tected by a large safety margin. Only in those instances in which the enzyme in question functions as part of the local control mechanism will the amount of enzyme locally present be expected to just barely suffice for the physiologic function to which it is related.

The safety margin of supply is well illustrated by the pharmacology of Diamox. The dose of this agent which is just sufficient to affect aqueous flow does not significantly influence gastric secretion. About 10 times the threshold dose for the eye is required to affect the stomach. More than 20 times the dose effective for the eye is required to seriously inhibit CO_2 loss from the lungs. Maren and Ballintine[6] find the concentration of Diamox slightly lower in the ciliary body than in the blood. Dosage of the inhibitor sufficient to cause a lowering of intraocular pressure does not suffice to produce severe respiratory acidosis. If the same enzyme is to be held responsible for both these effects it must be present in very much larger surplus in the red blood cell than in the ciliary body.

Knowing the time of blood transit through the lung, the rate of CO_2 discharge necessary to prevent respiratory acidosis can be estimated. A not unreasonable estimate is that the normal red blood cell content of carbonic anhydrase is several thousandfold in excess of the minimum required. Consequently, enzyme inhibition of upward of 99.9 percent needs to be achieved in order to produce a serious inhibition of pulmonary CO_2 output. Similar calculations cannot as yet be made for the ciliary body. However, at inhibitory dosage levels concentrations of the drug in the posterior chamber aqueous fluid of 0.5 to 1.0 gamma per cc. have been found by Maren and Becker.[7] The secretory organ cannot have been exposed to concentrations less than this. Dr. Maren[8] has found that a concentration of 0.004 gamma per cc. will inhibit the enzyme by approximately 50 percent in the test tube. It follows that at threshold dosage levels effective in lowering intraocular pressure the enzyme must be at least 98 to 99 percent inhibited. If carbonic anhydrase is a part of the intraocular secretory mechanism it is present in at least a 50-fold surplus—a large margin of safety but not as large as that in the red cell or possibly in the gastric mucosa.

2. Incomplete inhibition

Carbonic anhydrase is remarkable in that the chemical reaction which it catalyzes:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3$$

operates at a very appreciable velocity in the absence of the enzyme. If a physiologic function is dependent on this reaction, then it may be assumed that the physiologic function will not be completely inhibited even at dosage levels at which the enzyme is reduced to negligible activity. The renal effect of Diamox is an inhibition of the tubular reabsorption of sodium, but this inhibition is incomplete and is not enhanced above a given upper limit even by massive increase in the dosage of the inhibitor. If we apply this reasoning to the eye we shall expect that inhibition of flow will not be complete under the influence of this drug but should tend toward an upper limit with increasing dosage, corresponding to the minimum secretory rate that can be satisfied by the reaction rate of carbon dioxide hydration in the absence of the enzyme. Conversely we should expect that the influence of Diamox on intraocular pressure will be less if, for any reason, the rate of flow of the aqueous before presentation of Diamox is already slow.

Examples conforming to this expectation are to be found both in human and in rabbit physiology. In the first set of glaucoma patients initially studied by Dr. Becker,[5] it was found that glaucoma cases with intrinsically low rates of aqueous flow were relatively unresponsive to the drug. Using tonography as a basis for estimating the rate of flow, Dr. Becker has found that patients whose aqueous flow rates were under 1.0

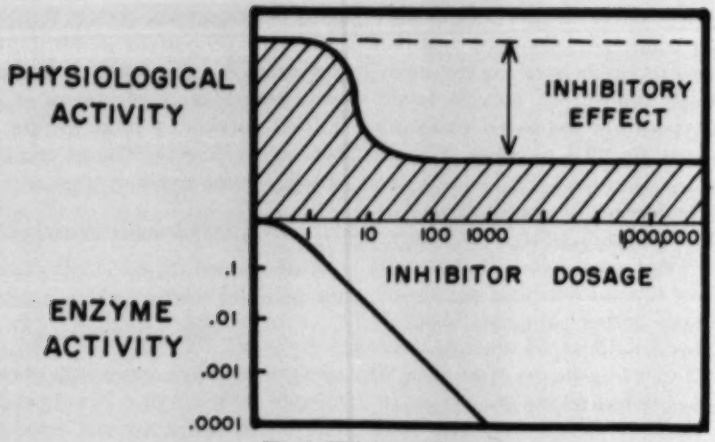

Fig. 1 (Friedenwald).

c.mm. per minute responded on the average very feebly to the drug.

In a recent study by Kupfer, Lawrence, and Linnér[9] on the long term control of glaucoma with Diamox it was shown that the rate of flow under Diamox averages 0.80 c.mm. per minute and that this rate is independent of the rate of flow before Diamox. It is plain that in man, roughly one third of the average normal rate of flow is independent of carbonic anhydrase activity.

Similar phenomena can be elicited experimentally in the rabbit. Dr. Kornblüth and Dr. Linnér[2] have shown that unilateral ligation of the common carotid in the rabbit results in an appreciable decrease in the flow of aqueous on the ligated side. Becker[10] has found that the administration of Diamox to such animals has its normal effect on the contralateral side but reduces the flow much less dramatically on the ligated side.

Moreover, among apparently normal rabbits one occasionally encounters an animal whose spontaneous flow rate at the time of observation happens to be far below the average normal. Such animals respond feebly or not at all to Diamox.[10] In the normal rabbit, on the other hand, the fully effective dose of Diamox results, on the average, in a 60 to 70 percent inhibition of aqueous flow.[11,*]

3. Intraocular pressure

If the flow of aqueous is greatly inhibited the intraocular pressure can be expected to fall close to the episcleral venous pressure. Linnér[2] has estimated this pressure as approximately 9.0 mm. Hg in the rabbit, 11 mm. Hg in recumbent humans.[12] This is,

* A rough estimate of the amount of surplus enzyme in the ciliary body may be reached as follows: The rate of secretion of intraocular fluid in rabbits proceeds at approximately one third of its normal rate when carbonic anhydrase is maximally inhibited. If we assume that this residual secretion rate is dependent upon the non-enzymatic hydration of CO_2, then the normal functioning requires enough carbonic anhydrase to triple the non-enzymatic reaction rate. Maren has defined one enzyme unit as that amount of enzyme which, when dissolved in 6.0 ml. doubles the rate of CO_2 hydration in the pH range of his test system. It is not certain that this ratio of enzyme catalyzed activity to uncatalyzed activity applies to physiologic pH range. With this reservation in mind, one can conclude that approximately two enzyme units per 6.0 ml. of secretary organ represent the minimum required for normal function. Ballintine and Maren find that the ciliary processes contain 10 to 20 enzyme units per gram, that is, 60 to 120 times the minimum estimated for full function. This argument presupposes a uniform distribution of the enzyme in the secretory organ—a highly unlikely assumption—but the agreement of this estimate with that given above, that is, that at threshold dose of Diamox the enzyme in the ciliary processes must be inhibited at least 98 to 99 percent, suggests that both estimates may be roughly correct.

under ordinary circumstances, the lowest pressure level that can be expected under the influence of Diamox. It follows that if before Diamox the intraocular pressure already approaches such a low level, any effect of Diamox in further reducing the pressure must be very small.

The intraocular pressure can be spontaneously low either because the rate of secretion of fluid is unusually slow or because the outflow resistance is unusually small. In the first instance, the animal may be classified as unresponsive to Diamox so far as aqueous flow is concerned. In the second, the Diamox effect on the rate of flow may be normal but the consequent change in intraocular pressure may be trivial. Thus the hypotensive effect of Diamox is much more dramatic in glaucomatous than in normal human eyes.

4. Homeostatic mechanisms

Many workers in the field of aqueous humor dynamics have given thought to the possible existence of homeostatic mechanisms stabilizing either the pressure or the flow in the eye. At the International Congress last autumn, Dr. Bárány[18] reported on some experiments suggesting that the resistance to outflow increased in response to diminished inflow. At the same meeting I[14] reported experiments with Dr. Linnér demonstrating a diminished rate of flow of the aqueous after intravenous injections of paraamino hippuric acid in rabbits. In spite of the diminished rate of flow the intraocular pressure in these animals did not fall appreciably. We concluded that the resistance to outflow must have increased and suggested that this might represent a normal compensatory or homeostatic mechanism in response to diminished inflow.

Similar increase in resistance to outflow occurs after carotid ligation. Kornblüth and Linnér[2] have shown that the inflow of aqueous is diminished after ligation but the pressure drops only slightly, resistance to outflow being increased.

If there is normally a homeostatic reflex increasing resistance to outflow in response to diminished rate of secretion of the aqueous we should be able to see this also after Diamox administration and this indeed proves to be the case. In man, following oral administration of 250 mg., there is a fall in intraocular pressure which, in nonglaucomatous eyes, usually reaches a maximum in three hours, begins to climb at six hours, and is back to the previous level by 10 hours. Tonography before and three hours after Diamox generally reveals a marked fall in rate of flow but no change in outflow resistance. On the other hand, Becker[10] has recently found that tonography at six to 24 hours after administration of Diamox shows a marked increase in resistance to outflow with only an incomplete recovery in the rate of inflow. The rise in pressure at the end of six hours is due to the compensatory outflow resistance long before the inhibition of secretion wanes.

Similar results can be obtained on animals. After intravenous injections of Diamox in the rabbit, responsive animals show a marked drop in intraocular pressure which reaches a minimum in 20 to 40 minutes. During the subsequent rise, as Becker and Constant[11] have found, the first change is often an increase in resistance to outflow which may long precede recovery of inflow. In some rabbits the compensatory increase in outflow resistance occurs so early that the drop in intraocular pressure is negligibly small in spite of a marked inhibition of inflow. These animals if measured only tonometrically would be classified as nonresponders. Tonographic measurements show that they are normally responsive with respect to inhibition of inflow, but supernormally responsive with respect to compensatory outflow resistance. In my own laboratory apparent nonresponding rabbits of this type have been rare, but in other laboratories they have been common. The mechanism by which the compensatory increase in outflow resistance is elicited and the mechanism by which this

compensatory response is suppressed is perhaps the most exciting current problem in this field. Open-angle glaucoma may, for instance, consist in an inability to suppress this enhanced resistance to outflow.

5. Effects of pH

The chemical reaction catalyzed by carbonic anhydrase takes place at a significant rate even in the absence of the enzyme. What, then, can be the physiologic function which the enzyme serves? The hydration of carbon dioxide and dehydration of carbonic acid are parts of the reaction by which carbon dioxide and bicarbonate participate in the acid base buffering of the tissues. Since chemical reactions within cells may generate or destroy acids or bases, and since the metabolic reactions tending to produce local tissue acidity or alkalinity may be very rapid, the presence of the enzyme carbonic anhydrase insures the rapid buffering of these reactions and hence may contribute to the stability of the tissue pH. This, at least, is a tenable hypothesis as to the role of this enzyme in some tissues.

Let us consider a tissue in which local metabolic processes produce acid and in which, if sufficient acidity is reached, some tissue function will be suppressed. Carbonic anhydrase will protect against this excess acidity. Inhibition of carbonic anhydrase will interfere with this buffering reaction and lead to inhibition of tissue function. If the carbonic anhydrase inhibitor is administered in the presence of a general acidosis the pharmacologic effect of the inhibitor will be facilitated. If the experiment is conducted in an alkalotic environment the drug will be antagonized. It is plain that acidosis or alkalosis may be expected to facilitate some Diamox effects and to antagonize others.

This indeed has been found to be true. In the kidney, Diamox produces an alkaline diuresis. The diuresis may be facilitated by administration of sodium bicarbonate. It can be suppressed by administration of ammonium chloride. Diamox is an anticonvulsant. This effect is facilitated by administration of ammonium chloride. If current theories regarding the role of carbonic anhydrase in the stomach are correct the inhibitory effect of Diamox on this organ should be facilitated by alkalosis, antagonized by acidosis. It is plain that if the effect of Diamox on the intraocular pressure is via an inhibition of carbonic anhydrase, the Diamox effect should be sensitive to acid-base disturbances.

I have elsewhere[15] developed the rather complicated expectations which the redox theory of aqueous secretion impose on these questions and shall not repeat them here. Experimental tests of this combined hypothesis have not been completed. Irrespective of the redox hypothesis, however, it may be pointed out that in some patients the ocular response to Diamox may be facilitated by concurrent administration of ammonium chloride.

6. Alternative hypotheses

I have enumerated five different ways in which the effect of Diamox on intraocular pressure may be facilitated or antagonized. These considerations were arrived at by assuming that Diamox affected intraocular pressure through inhibition of carbonic anhydrase. All of the various expectations that have been built on this concept and that have been tested experimentally have so far proved to be true. An hypothesis of physiologic mechanism is, however, never conclusively proved. The most that can be expected is that the hypothesis is not contradicted by any known facts and that it leads to new experiments and new confirmations.

It would appear that the hypothesis I have outlined satisfies these criteria. Recently, however, Green and Leopold have reported results in apparent contradiction. It is necessary to examine their data and arguments in some detail. They report that at dosage levels of Diamox under which 93 percent of the carbonic anhydrase activity of the

anterior uvea is inhibited[16] no fall in intraocular pressure was recorded in their animals.[17] In view of the surplus carbonic anhydrase in other organs there is no a priori reason to believe that 93-percent inhibition in the ciliary body would result in physiologic effects. I have already indicated that the data of Ballintine and Maren[6] suggest upward of 50-fold excess of the enzyme in the ciliary body. On the other hand, in view of the limits of sensitivity of the method, too much reliance should not be placed on the estimate of seven percent remaining enzymatic activity. The percent inhibition may well have been considerably higher than that estimated.*

The dosage used in these experiments was that which other workers have found effective in lowering intraocular pressure in rabbits. Green, Leopold, et al.,[17] on the other hand, claim that on this dose of Diamox the intraocular pressure of rabbits does not fall. To reach this conclusion they have invented a rather entertaining but highly inappropriate method of statistical analysis which they have applied to their data. Those of us who have dealt with "responsive" rabbits have found—and Becker[11] has described in detail—that after an effective intravenous dose of Diamox in a rabbit the intraocular pressure falls rapidly, reaching a minimum in 20 to 40 minutes, begins to recover by one hour, and is often almost fully recovered by two hours after the injection. Green and Leopold made tonometric measurements before, and 30, 60, 120, and sometimes 180 minutes after Diamox injection. Instead of observing the course of the intraocular pressure as exemplified by their data, they fitted a straight line by an equivalent of the least squares procedure to each set of observations and recorded the slope of this line. Since most of their observations were during recovery, one would expect an upward slope of the line and this is indeed what they found. However, since the initial fall is mixed in with the recovery the upward slope of recovery is largely obscured and in their analysis does not reach statistical significance. In any case this is hardly the way to test whether or not a fall in pressure occurred during the first 30 minutes as was to be expected from the reports of others. I have analyzed the data and find that between zero and 30 minutes in their experiments the intraocular pressure fell on the average the equivalent of 1.5 Schiøtz scale units and that this fall is statistically significant by odds of more than 100 to 1. This effect is, of course, a small one. In maximally responsive rabbits the change in the Schiøtz reading is two to three times the average for the Philadelphia rabbits, but the presence of a feeble response is not proof of the absence of a response.

In their third paper[18] the authors claim that with the same standard dose of Diamox they are unable to demonstrate a fall in aqueous bicarbonate. In this case, the fault is not with their statistics but with the plan of their experiment.

Many authors have shown that if one alters the secretory composition of the aqueous, as for instance, by injecting some test substance intravenously, then an appreciable time is required before a steady state concentration with respect to the test substance is reached in the anterior chamber. Kinsey[1] has, in fact, used the dynamics of this approach to a new composition in posterior and anterior aqueous as a means of estimating the rate of aqueous flow. It is possible to reverse Kinsey's argument and to calculate

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* The technical procedure used by Green et al. consisted in diluting the ground-up anterior uvea of one rabbit eye into a final volume of over 2.0 ml. The concentration of the inhibitor in the titration chamber is therefore much less than that originally present in the tissues and an underestimate of the in vivo degree of inhibition is to be expected. On the other hand, Green et al. have at times reported "complete" inhibition of carbonic anhydrase in some experiments. This must be taken to mean merely that within the limits of sensitivity of their method no enzyme activity could be measured. Total inhibition of an enzyme by a dissociable inhibitor can be postulated only with infinite concentration of the inhibitor.

that if Diamox slows the aqueous flow by 60 percent as Becker finds and if it does not change the diffusion coefficient for bicarbonate across the anterior chamber blood barrier, then, in 30 minutes somewhere between 15 percent and 20 percent of the maximum Diamox induced bicarbonate fall in the anterior chamber is to be expected.

Becker finds that after six hours of continuous Diamox administration the anterior chamber bicarbonate falls by about six millimols. The expected fall at 30 minutes is, therefore, less than 1.5 millimols. Green and Leopold actually found an average fall of one millimol but owing to large experimental scatter and limited number of experiments this figure is not statistically significant. To have found almost precisely what a theory predicts but to have done insufficient experiments to make this finding statistically significant hardly constitutes the refutation of a theory—nor is there any good sense in attempting to refute a theory by choosing an experimental plan under which the effect to be sought for is expected, by the theory under attack, to be so small as to be measured only with great difficulty.

In their report presented in this symposium Green and Leopold have added some new data to the universal finding that subconjunctival administration of Diamox is largely ineffective. What should be remembered in this connection is that the secretory organ as a consequence of its own physiologic activity is constantly being washed by fresh fluid derived from the plasma. It is, therefore, the one intraocular structure most difficult to contaminate with subconjunctivally injected test substances. There is no reason why adjacent nonsecreting tissues may not be heavily loaded with the inhibitor and that this load may not be sufficient to inhibit the enzyme in the ground-up tissue used to titrate enzyme activity. The authors are aware of this uncertainty and point out that their experiments do not prove inhibition of the enzyme in the secretory organ in vivo.

Summary

If I may summarize once more—theories of physiologic or pharmacologic mechanisms are never proven. They justify themselves if they are not contradicted by any known facts and if the expectations based on these theories are confirmed by new experiments. In this respect, one can conclude that current knowledge is fully in accord with the hypothesis that Diamox exerts its pharmacologic effect on intraocular pressure by inhibiting the enzyme carbonic anhydrase and that this enzyme facilitates the normal inflow of fluid into the eye.

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DIAMOX AND INTRAOCULAR FLUID DYNAMICS*

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According to the secretion-diffusion theory of the formation of aqueous humor, water and solutes enter the anterior chamber both by diffusion through the blood vessels of the iris and by flow from the posterior chamber. The aqueous humor of the posterior chamber, which differs in composition from that in the anterior chamber, is similarly formed in part by diffusion and in part by mechanisms requiring the expenditure of energy (secretion).

It is believed that bicarbonate ions, especially, are involved in the secretory process. These ions are produced in the ciliary processes whence they diffuse into the posterior chamber. Simultaneously, hydrogen ions are lost to the blood thus leaving the bicarbonate ions momentarily electrically unbalanced which leads to the energetic transfer of cations, chiefly sodium, into the posterior chamber. Other anions (except ascorbate) and nonelectrolytes are presumed to enter the posterior chamber by passive diffusion only.

The aqueous is a fluid which, primarily because it contains a greater concentration of bicarbonate and sodium than plasma, is hypertonic to the blood; this condition results in the osmotic transfer of water from plasma to posterior aqueous humor. The net transfer of water leads to a through and through flow of aqueous humor from posterior chamber to the anterior chamber and eventually out at the angle.

The reactions involved in the production of bicarbonate ions in the ciliary processes are thought to depend, as they do elsewhere in the body, on the action of the enzyme carbonic anhydrase:

$$H_2O + CO_2 \overset{\text{C.A.}}{\rightleftharpoons} H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

The availability of Diamox, which is known to inhibit carbonic anhydrase, makes it possible to test some of the basic tenets of the secretion-diffusion theory. For instance, it would be expected that (1) the concentration of bicarbonate in the aqueous humor would be decreased; (2) flow would be reduced, presumably as a result of lowered bicarbonate; (3) the concentration of other solutes such as chloride and also ascor-

* An abstract of the paper presented before the Association for Research in Ophthalmology, June, 1955.

bate would rise owing to a diminished dilution effect as a result of the reduced amount of water entering the posterior chamber; and (4) the turnover rate of sodium would decrease.

Observations made independently by Dr. Becker and us show that the concentrations of bicarbonate in the aqueous humor of both the anterior and posterior chambers are reduced, and those of ascorbate and chloride are increased after administration of Diamox to rabbits. Aqueous flow was found, using various methods, to be decreased by this drug. Dr. Becker and Dr. Grant employed tonography, Dr. Friedenwald and Dr. Linnér, delay in fluoroscein appearance time, and we, the para-aminohippuric-acid disappearance time.

These results are compatible with the notion not only that flow is dependent on production of bicarbonate, but that the mode of action of Diamox in the eye is through the local inhibition of carbonic anhydrase. Apparently incompatible with the secretion-diffusion theory, as outlined above, is our observation that the turnover rate of sodium in the posterior and anterior chambers is not appreciably reduced by Diamox. This recent finding indicates a need for re-evaluating the secretion-diffusion theory of aqueous humor formation, particularly as it pertains to mode of entrance of sodium, and/or re-evaluating the mode of action of Diamox in reducing flow.

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CARBONIC ANHYDRASE ACTIVITY AND THE DISTRIBUTION OF DIAMOX IN THE RABBIT EYE

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A theory for the formation of aqueous humor supposes that the production of bicarbonate ions in the posterior chamber is a primary event.[1] Dependence of such formation on carbonic anhydrase is inferred by the occurrence of this enzyme in the anterior uvea[2] and the lowering of the intraocular pressure of glaucomatous patients by the specific carbonic anhydrase inhibitor acetazoleamide (Diamox‡).[3]

The lowering of intraocular pressure in albino rabbits by intravenous administration of Diamox is variable. Green et al.[4] interpreted their data as showing no lowering of pressure, Kinsey[5] found reduction of pressure in only a small proportion of rabbits, while Becker[6] found reduction in almost all.

Our supply contained a few rabbits (hereafter referred to as "reactors") which responded to each administration of Diamox with a profound fall in intraocular pressure, some (referred to as "nonreactors") in which intraocular pressure was relatively unaffected, and many in which repeated trials produced equivocal results.

The purposes of this investigation were (1) to localize further the site of carbonic anhydrase activity in the eye, (2) to determine the distribution of Diamox in the eye, and (3) to find if enzyme activity or drug distribution differed between reactors and nonreactors. Particular attention was given to quantitative estimation and correction for blood in tissues, a point of particular im-

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‡ 2 acetylamino, —1, 3, 4, thiadiazole, —5 sulfonamide.

portance because of the high concentration of both enzyme and inhibitor in erythrocytes.

Methods

Lots of six to 12 albino rabbits (New Zealand white giants) weighing 3.0 to 5.0 kg. were obtained from a single supplier. From March 31, 1954, to January 1, 1955, they were maintained on Purina Rabbit Pellets and from January 1, 1955, to May 8, 1955, on Rockland Rabbit Ration.

Forty-six rabbits were tested to determine if an intravenous injection of the sodium salt of Diamox* would produce a fall in intraocular pressure. In each test control pressures were measured, the Diamox was injected in the marginal ear vein, and the pressures measured at uniform intervals (10, 15, or 20 min.) for one and one-half to three hours. On most of the rabbits the test was repeated one or more times three or more days after the previous test. The same certified Schiøtz tonometer was used for all pressure measurements.

Each rabbit also had the test repeated one or more times using, instead of Diamox, sodium sulfadiazine in approximately equal molar concentration. These tests were controls to evaluate the effects of handling, injection, and repeated tension measurements.

Preliminary tests showed that the response of a rabbit was not changed enough by changing the interval between tension measurements (10, 15, or 20 min.) to affect its classification as a reactor or nonreactor. Doses of Diamox were 10 and 20 mg./kg. (solutions of 5.0 and 10 mg./ml.). Preliminary tests showed that the pressure changes were the same for doses of 10 and 20 mg./kg.

When enzyme activity was determined, blood was obtained by cardiac puncture with a heparinized syringe, the hematocrit determined, and a specimen of plasma obtained by centrifugation. The rabbit was decapitated and aqueous humor collected. The eyes were enucleated, washed in iced Krebs Ringer solution, and opened equatorially. The lens and vitreous were removed from the anterior half and the anterior half everted on a special holder in a bath of iced Krebs Ringer solution or normal saline. Under a dissecting microscope the individual ciliary processes were dissected from the underlying iris and the iris was removed.

The ciliary processes and irises were weighed, then homogenized in ice cold glass homogenizers with enough water to give a 1.5, 2.0, or 4.0-percent homogenate. The amount of blood in a portion of the homogenate was determined by the pseudo-peroxidase reaction (modified from Bing and Baker[7]) with purified benzidine in a Klett photoelectric colorimeter using the rabbit's blood as a standard (see appendix).

The homogenates, aqueous humor, blood, and plasma were frozen and packed in "dry ice" in a Dewar flask and shipped via air express to Stamford, Connecticut, where they were assayed for carbonic anhydrase activity by the changing pH method.[8] The results were expressed in enzyme units as defined in reference[8] and were corrected for the carbonic anhydrase activity of the red blood cells present. In experiments to determine the concentration of Diamox, the procedure was changed only in that control blood was obtained by cardiac puncture, then Diamox was administered intravenously, and a second blood specimen obtained just before decapitation.

In an attempt to reduce the correction for blood retained in ciliary processes, two rabbit heads (Nos. 572, 576) were perfused through both carotids with 1,500 ml. of iced Krebs Ringer solution each, and the dissections were then performed as described above. In these dissections, the ciliary processes appeared to be almost completely free of blood.

Two eyes for enzyme activity (No. 559) and four for Diamox distribution (No. 1, 562) were dissected in a cold room in air,

* Prepared by addition of 1.6 moles of sodium hydroxide to one mole of Diamox. "Diamox" refers to such a solution throughout.

without separating ciliary processes from iris, to determine if enzyme or Diamox was washed out by dissection in the bath. The enzyme activity of homogenates of tissues obtained after Diamox administration was measured in six eyes (Nos. 479, 503, 514).

Results

Forty-six rabbits were tested 123 times with Diamox and 63 times with sulfadiazine with the results shown in Table 1.

The probability of the differences between the means for the two substances occurring by chance is less than 10^{-8}. The probability of the differences between the standard deviations for the two substances occurring by chance is less than one percent ($P < .01$).

In repeated tests with Diamox, certain rabbits showed increases in tonometer readings that were consistently greater than in others in which the increases were small or erratic. Since the average fall produced by sulfadiazine injection, plus two standard deviations equals 3.8, rabbits were called "reactors" if, on every test with Diamox, the tonometer reading rose four or more units. Since the tonometer reading after Diamox rarely exceeded 8.0 and because a few rabbits had control readings of 4.0 or more, rabbits were also called reactors if the tonometer reading consistently rose three units and the highest reading was 7.5 or more. If the rise in tonometer reading was consistently less than these limits, the rabbit was called a "nonreactor."

By these criteria six were reactors and 19 were nonreactors. Fifteen could not be classified because their responses were erratic; on some tests being reactors, on others nonreactors, and frequently showing different responses in the two eyes on the same test. (Of the 19 nonreactors, seven were tested only once with Diamox and might have been erratic on repeated tests.) Four rabbits which were reactors initially became nonreactors and two which were initially nonreactors, became reacters when observed over periods of one to four months.

Rabbits were selected from the groups of reactors and nonreactors for carbonic anhydrase and Diamox assays, the results of which are shown in Tables 2 and 4. All values are corrected for the blood content of the tissue. The blood content and the total enzyme activity of the tissues and the enzyme activity of the contained blood are shown in Table 3. The corrections in the Diamox assays for the inhibitor in the blood in the tissues are insignificant.

TABLE 1

Results of tests with Diamox and sulfadiazine

Test Substance		Mean Maximum Increase in Tonometer Scale Reading ±Standard Deviation
Sulfadiazine	O.D.	1.8±1.0
	O.S.	1.8±1.0
Diamox	O.D.	3.0±1.4
	O.S.	3.0±1.3

Discussion

These findings (table 2) confirm the known carbonic anhydrase activity of the lens.[9] Because there were so few reactors among these rabbits, there are not enough data to be certain, but there is no obvious difference in enzyme activity between tissues from reactors and nonreactors. The concentration of carbonic anhydrase in the ciliary processes is approximately three times that in the iris.

An average of 0.012 gm. of processes and 0.052 gm. of iris was obtained from one eye. The average concentration of enzyme in the processes was 25 units/gm. and in the iris, 9.0 units/gm. The sum of the enzyme activity of iris and ciliary processes is therefore 0.8 enzyme units. The weight of undissected anterior uvea average 0.060 gm. In the experiment where the tissues were not washed the combined iris and processes had 23 E.U./gm. so that the total activity of the anterior uvea of one eye was 1.3 E.U.

Wistrand[9] reported that the carbonic an-

TABLE 2

CARBONIC ANHYDRASE ACTIVITY OF OCULAR TISSUES

Rabbit No.	Response to "Diamox"	Carbonic Anhydrase Activity. Enzyme Units/Gm. of Tissue				
		Whole Blood	Lens	Ciliary Processes	Iris	Aqueous Humor
1A pooled 2A specimens	Not tested	330	270	39.0	<5	
723	Reactor	360	215	47.0	<25*	<10*
573	Reactor	670		5.0	4	
493	Nonreactor	550	175	18.5	15	<2
494	Nonreactor	525	102	18.0	5	
496	Nonreactor	388	147	pooled specimens		
576 {perfused heads}	Nonreactor	800		25.0	15	
572 {perfused heads}	Nonreactor	730		8.0	9	
559 Dissection in air	Equivocal	750	265	intact iris and processes		
				O.D. 24	*O.S.* 22	

* Insufficient material for further assay.

hydrase activity of the combined anterior uvea was one seventh that of erythrocytes. In our experiments the average activity of the erythrocytes was 567 units so that at the most the activity of the anterior uvea was not more than one twentieth that of the erythrocytes. Even if the correction for erythrocytes in the tissue is omitted in our highest values, the calculated activity of the anterior uvea is only 25 E.U./gm. or about one twenty-third that of erythrocytes. The data of Green et al.[10] are of such form that they cannot be compared to our results.

Table 4 indicates that there is no obvious difference in uptake of Diamox by ocular tissues of reactors and nonreactors. There

TABLE 3

CORRECTION FOR BLOOD CONTENT OF TISSUES

Rabbit No.	Tissue	Erythrocyte Content gm./gm.	Gross Carbonic Anhydrase Activity Enzyme units/gm.	Correction for Blood Enzyme units/gm.	Net Enzyme Activity Enzyme units/gm. (Table 2)
1A 2A	Ciliary processes	0.0013	40.0	1.0	39.0
723	Ciliary processes	0.025	70.0	23.0	47.0
573	Iris	0.002	7.0	3.0	4.0
	Ciliary processes	0.006	15.0	10.0	5.0
493	Iris	0.004	20.0	5.0	15.0
	Ciliary processes	0.026	50.0	32.5	18.5
494 496	Ciliary processes	0.010	30.0	12.0	18.0
576*	Iris	0.0005	16.0	1.0	15.0
	Ciliary processes	0.001	27.0	2.0	25.0
572*	Iris	0.001	11.0	2.0	9.0
	Ciliary processes	0.004	15.0	7.0	8.0
559	Undissected Anterior Uvea				
	O.D.	0.0006	25.0	1.0	24.0
	O.S.	0.001	23.0	1.0	22.0

* Heads perfused.

TABLE 4

DIAMOX CONCENTRATION IN OCULAR TISSUES

Rabbit No.	Dose of "Diamox" (mg./kg.)	Response to "Diamox"	Interval* (min.)	"Diamox" Concentration (microgram/gram)						
				Lens	Aqueous Humor	Whole Blood	Ciliary Processes	Iris	Plasma	Aqueous Plasma Ratio
787	25 i.v. 25 i.p.	Nonreactor	120	0.8	0.6		2.2		12.0	0.05
574	20 i.v. q. 30 min ×3	Nonreactor	120	0.7	0.9	18.0	5.0	1.3	16.6	0.054
566	20 i.v.	Nonreactor	45	<0.2		13.5	2.9	0.6	10.2	
499	20 i.v.	Reactor	20	0.8	0.3	20.8	7.2	1.3	19.5	0.015
507	20 i.v.	Nonreactor	60	<.7	0.4	8.2	5.2	1.5	9.2	0.043
479	10 i.v.	Reactor	30	1.2	0.5	10.3			8.5	0.058
503	10 i.v.	Nonreactor	20	2.0		8.2			9.0	
565	20 i.v.	Reactor Dissected in air	30	0.2	0.8	29.5	intact iris and processes *O.D.* 11.7	*O.S.* 12.1	23.5	0.035
1	20 i.v.	Dissected in air	60	0.0	0.8	18.5	2.4	2.2	12.2	0.065

* From first Diamox injection to decapitation.

was about four times as much Diamox present in the ciliary processes as in the iris indicating some localization at the presumed site of action. The aqueous/plasma ratios agree closely with spinal fluid/plasma ratios found previously.[11]

The concentration of Diamox in aqueous humor ranged 0.3 to 0.9 γ/ml. These data do not agree with those of Green[10] who reports a range of 0 to 23 γ/ml. for similar experiments using a spectrophotometric assay. Green also reported concentrations of Diamox in the uvea 20 times those reported here.

Table 5 gives the apparent enzyme activity in vitro of various tissues following administration of Diamox. The data reflect the interaction of enzyme and inhibitor in the

TABLE 5

APPARENT ENZYME ACTIVITY IN TISSUES OBTAINED AFTER DIAMOX ADMINISTRATION

Rabbit No.	Dose of Diamox mg./kg.	Interval Min.[1]	[2]	Blood	Plasma	Lens	Ciliary Processes[3]	Iris	Aqueous Humor	Control Blood (No Diamox)
479	10	30	Activity	312.0		135.0	<13	<5		650
			Diamox Conc.	10.3	8.5	1.2			0.53	0
503	10	20	Activity	250.0			<27			408
			Diamox Conc.	8.2	9.0					0
514	20 q. 30 min. ×3	120	Activity	160.0		274.0		0		
			Diamox Conc.	18.0	16.6	0.7	5.0	1.3	0.9	0

[1] From Diamox administration to decapitation.
[2] Activity = Apparent enzyme units/gm. Diamox Conc. = micrograms/gm.
[3] Insufficient material to detect any lower enzyme activity.

dilute in vitro system, but may not mirror in vivo events since dilution and disruption of cellular organization may change enzyme inhibitor relation from what it was in the body. The value of the data lies in the qualitative comparison among tissues with greatly differing concentrations of enzyme and drug, as were noted in Tables 2 and 4.

In blood, with very high enzyme concentration and high Diamox concentration, there was some decrease in enzyme activity, confirming previous work in dog.[11] In the lens, with high enzyme concentration and very little Diamox, effect on enzyme activity was imperceptible. In the iris with relatively low enzyme activity and moderate Diamox concentration no carbonic anhydrase activity was detectable.

Insufficient material was available to demonstrate the extent of the inhibition in the processes except that, as noted, no enzyme activity was detected as far as it was sought (table 5). However, the order of such inhibition may be estimated since we know the concentration of inhibitor present (tables 4 and 5, about 5 γ/gm.) and the concentration of enzyme (table 2, about 20 units/gm.). In the in vitro system used (ref. 8, fig. 2), 0.006 γ Diamox/ml. inhibits 50 percent of enzyme activity. Further investigation showed that 0.05 γ/ml. abolishes all detectable enzyme activity. There is then in the ciliary process about 100 times as much Diamox as needed for "complete" inhibition. This figure is admittedly inaccurate since there is considerably more enzyme in vivo than in vitro and there is some evidence that increased concentration of enzyme does decrease the degree of inhibition in this system. Nevertheless, the 100-fold margin does allow the reasonable possibility that virtually complete inhibition of carbonic anhydrase is achieved in the ciliary process.

Summary

(1) In a series of 46 rabbits given 10 or 20 mg./kg. Diamox intravenously on 123 occasions, the average increase in Schiøtz tonometer reading was 3.0 (standard deviation = 1.3). When the same rabbits were given sulfadiazine in equal concentration the average increase was 1.8 (S.D. = 1.0).

(2) Six rabbits had consistently large increases in tonometer readings and 19 had consistently small increases.

(3) There were no obvious differences in either carbonic anhydrase activity or concentration of Diamox in tissues from rabbits that had large and small increases in tonometer readings after Diamox.

(4) The amount of Diamox present in iris and in ciliary processes after intravenous administration of Diamox (10 or 20 mg./kg.) is enough to inhibit almost completely carbonic anhydrase activity, if the inhibitor reaches the enzyme. There was insufficient Diamox present in the lens to have detectable effect on its carbonic anhydrase activity.

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Appendix A

Determination of the blood content of homogenates by the pseudoperoxidase reaction

The sensitivity and reproducibity of analyses for blood by the pseudoperoxidase reaction depend largely upon the purity of the benzidine and the standardization of the hydrogen peroxide (Bing and Baker[7]).

In agreement with Bing[10] we found that two lots of commercial benzidine were unsuitable unless purified by the method of Bing.[12] The unpurified material frequently gave reagent blanks which were a large and variable fraction of the total color density produced by the homogenates. When an attempt is made to compare the homogenates to specimens of diluted blood by schemes involving serial dilution, the color from the reagents may mask the color from the blood.

Therefore the method of Bing and Baker,[7] using benzidine reagent purified and prepared according to Bing,[12] was modified as follows:

The hematocrit (H) of the rabbit's heparinized blood was determined. Blood was diluted one part to 20,000 and 0.1, 0.3, and 0.5 ml. transferred to colorimeter tubes and made up to 0.5 ml. with water. The homogenates were diluted with water

so that there was one part tissue in 50 to 350 parts of homogenate ($\frac{W}{V} = \frac{1}{50}$ to $\frac{1}{350}$). From 0.1 to 0.3 ml. (V) of the diluted homogenate was measured into a colorimeter tube and made up to 0.5 ml. with water. Water, 0.5 ml., was placed in a colorimeter tube for a reagent blank.

To each colorimeter tube 1.0 ml. of the benzidine reagent was added and mixed; then 0.5 ml. of 0.6-percent hydrogen perioxide was added and mixed. After one hour 12.0 ml. of 20-percent acetic acid was added and the color measured in the Klett-Sommerson colorimeter using a No.-50 filter. When the volume of diluted blood was plotted against the Klett readings, a straight line resulted. From this graph the volume of diluted blood (D) equivalent to the volume (V) of dilute homogenate in the colorimeter tube was found.

If the concentration of tissue in the homogenate is $\frac{W}{V}$ gm./ml. and g is the gm. of erythrocytes per gm. of tissue, $g = \frac{DHV}{20{,}000}\left(\frac{V}{W}\right)$.

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Discussion

Dr. John E. Harris (Portland, Oregon): I certainly feel that we owe the essayists a heartfelt vote of thanks for their tremendous contributions.

They have raised several intriguing questions. Among them is the problem of the rabbits that do not respond to Diamox.

As I recall, Dr. Becker, you found that the "nonreactors" among the rabbits showed a diminished facility of outflow and felt that this was the reason for their poor response to Diamox. Is that correct?

Dr. Becker: The largest number of them.

Dr. Harris: In a paper recently published by Dr. Kinsey and his associates, it was demonstrated that the "nonreactors" could in certain cases be converted to "reactors by increasing the salt content of the diet or by administering aldosterone. You mentioned that 9-a-fluorohydrocortisone was also effective. Both of these hormones are potent sodium retainers. One can conclude, therefore, that such treatment must increase the facility of outflow in those "nonreacting" animals under the conditions of the experiment. I am wondering whether you have found this to be so, and, if so, whether you have any thoughts on the mechanism by which this effect might be mediated?

I do not know that we can dismiss the experiments of Dr. Green and his associates quite so readily as has Dr. Friedenwald. There are certain discrepancies among the accumulated data which must, of course, be resolved by further experimentation. Among these is the question of whether systemic administration of Diamox reduces the bicarbonate concentration of the aqueous independently of the changes in the plasma. There is almost universal agreement, however, that while systemic administration of the drug lowers the ocular tension in both glaucomatous eyes and in the normal eyes of many rabbits, it is singularly ineffective when administered locally by a variety of routes. The observation that subconjunctival injection inhibits carbonic anhydrase activity of the

anterior uveal tissue is therefore significant. As Dr. Friedenwald has mentioned, considerable reduction in enzyme concentration might cause only a slight alteration of cellular function. Dr. Green and his co-workers were measuring carbonic anhydrase activity and presumably, therefore, biologic function, insofar as we can project such in vitro experiments to in vivo conditions. If the hypotensive action of the drug is as postulated, a decrease in ocular tension following subconjunctival injection would reasonably be predicted from the work of these authors.

Tissue organization (and even cellular organization) undoubtedly plays an important role in secretory function, however, and, as has been pointed out, the side to which an inhibitor is delivered must unquestionably be significant. This has been demonstrated with other drugs. For example, the particular enzyme which the mercurial diuretics inhibit is widely distributed in the body. Nevertheless, these drugs apparently concentrate only in the kidney tubule, where they inhibit enzyme function and thus promote diuresis but do not alter the activity of the same enzyme elsewhere. I have wondered whether Diamox may be similarly concentrated at certain strategic points, since in spite of the widespread distribution of carbonic anhydrase, the drug seems preferentially to affect the kidney tubule (which may result simply from its concentration by the urinary tract), the ciliary body, and probably the choroid plexus.

The final answer, I think, is still unknown.

Dr. Bernard Becker (St. Louis, Missouri): We have experiments in progress to determine what substances like 9-alphafluorohydrocortisone do to facility of outflow. All I can say is that there is suggestive evidence that, in animals where the facility of outflow is decreased, either spontaneously or because of previous Diamox administration, 9-alphafluorohydrocortisone improves that facility of outflow.

This may not be a means of improving facility of outflow in all animals. It may be a permissive effect, if you like, a means of blocking the reflex of closure of outflow channels. This is purely speculative at present, however.

THE EFFECT OF SYMPATHETIC NERVE IMPULSES ON THE CILIARY MUSCLE*

Carlton E. Melton, Ph.D., Edward W. Purnell, M.A., and Gerhard A. Brecher,† M.D.
Cleveland, Ohio

From time to time during the past century reports have appeared in the literature either claiming or denying sympathetic innervation of the ciliary muscle (Fleming[1]). Attention has been focused again on the sympathetic innervation of structures within the eye because of the role which this innervation may play in aqueous drainage and chronic simple glaucoma. In view of the confusion surrounding this subject, it appeared necessary to obtain basic information, by direct means, about the mode of action of the sympathetic nervous system on the intraocular musculature, specifically the ciliary muscle.

With the exception of some controversial older reports, no information is available regarding the direct action of sympathetic excitation on the ciliary muscle. Cogan[2] has reviewed the early literature and has presented his own clinical observations on Horner's syndrome. He showed that a decrease of accommodative power amounting to 0.5 to 2.5 diopters was sympathetically mediated. Cogan expressed the view that, in the human, the radial fibers of the ciliary muscle were sympathetically innervated and that contraction of these fibers resulted in flattening of the lens.

Following Cogan's excellent review, Olmsted[3-7] and his collaborators investigated the effect of sympathetic stimulation on the accommodative power of the eyes in cats, rabbits, dogs, and monkeys. In all species ex-

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amined, they found that stimulation of the cervical sympathetic trunk caused flattening of the lens. The nerve fibers responsible for lens flattening had the same origin and course as those effecting pupillary dilatation. Furthermore, extirpation of the superior cervical ganglion caused myopia on the operated side and section of the third nerve caused ipsilateral hypermetropia.

Siebeck[8] presented good evidence that in the human eye the rest-point of accommodation is between the far point and the near point. Sympathomimetic drugs instilled into the conjunctival sac shifted the rest point toward the far point. Siebeck concluded that Helmholtz's theory of distance accommodation explained only the dioptric changes between the near-point and rest-point. However, the remaining one or two diopters of distance accommodation could not be explained by the Helmholtz theory because, in his opinion, they were sympathetically mediated.

Using enucleated cats' eyes, Meesmann[9] demonstrated that parasympathomimetically-induced contractions of the ciliary muscle could be partially or entirely counteracted by sympathomimetic agents. He concluded that the ciliary muscle was dually innervated. This view was supported by the histologic work of Wolter,[10] according to whom the ciliary muscle is supplied by two types of nerve fibers, one of which he believed to be sympathetic in character.

From the evidence in the literature, it appears that distance accommodation can be brought about by sympathetic nerve impulses. However, the site, type, and nature of the sympathetically innervated end organ responsible for the effect is still obscure. Fleming[1] suggested that the myopia resulting from superior cervical ganglionectomy was due to an engorgement of the ciliary body with blood. According to this hypothesis, such engorgement caused an increase in volume of the ciliary body and a consequent decrease in tension on the zonule. He arrived at this conclusion because the time course during which the myopia subsided after several days paralleled that during which vascular dilatation subsided in other organs after sympathectomy. Conversely, Fleming thought that stimulation of the cervical sympathetic trunk resulting in flattening of the lens by constriction of the ciliary vasculature which caused a decrease in volume of the ciliary body and tightening of the zonule.

Obviously, the action of the sympathetic nervous system on the ciliary muscle could not be elucidated by adding further circumstantial evidence or by a reinterpretation of the known data. Experiments were therefore designed which would yield direct information about ciliary muscle movements under strictly controlled conditions in the enucleated eye with all circulatory and extraocular effects excluded.

Method

All experiments were performed on cats. The eyes, with their autonomic nerves intact, were excised according to the following procedure. The orbit was exenterated and the eye and its adnexa mounted on a paraffined block. Extraneous tissue was carefully removed and the ciliary ganglion and the long and short ciliary nerves were isolated. The ciliary muscle was exposed through a meridional scleral window which began one to two mm. back of the limbus and measured about 2.0 to 9.0 mm. Two pairs of silver-wire stimulating electrodes mounted on manipulators were brought into position; one pair was placed either on the ciliary ganglion or short ciliary nerves and the other pair on one of the long ciliary nerves. Electrical stimuli were delivered by either an inductorium or an electronic square wave generator. By means of a switch, stimuli could be directed to either set of nerves or to both simultaneously. A 6-0 silk loop was passed loosely through the superficial layers of the ciliary muscle and attached to the plate pin of an RCA No. 5734 mechano-electronic vacuum tube transducer. The output of the tube was amplified by a Brush D-C amplifier

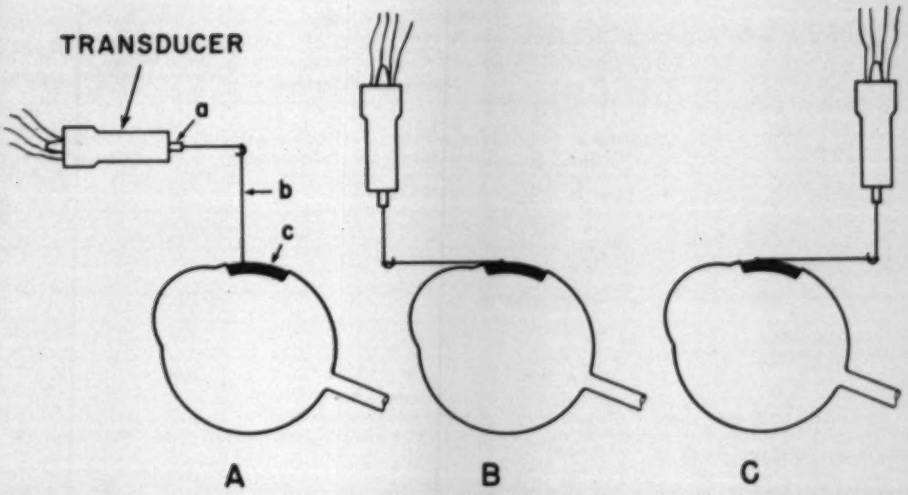

Fig. 1 (Melton, Purnell and Brecher). Diagram illustrating different directions in which the movement of the ciliary muscle was transmitted to the plate pin of the transducer. (A) Recording in radial direction. (B) Recording in tangentially forward direction. (C) Recording in tangentially backward direction. (a) Plate pin of transducer tube. (b) 6-0 thread loop. (c) Scleral window.

and fed onto the pen of a single channel Brush ink-writer. The preparation was bathed by a continuous stream of Ringer solution kept at 38°C.

Records were taken in three directions from the same point on the muscle (fig. 1): radially (A), tangentially forward (B), and tangentially backward (C). An increase in tension on the plate pin of the transducer tube was recorded as an upward deflection of the recording pen; a decrease in tension was recorded as a downward deflection. The tension in milligrams developed by the muscle was determined by hanging weights on the pin of the transducer.

Results

Stimulation of the sympathetic (long ciliary) and of the parasympathetic nerves (ciliary ganglion or short ciliary nerves) always caused marked movement of the ciliary muscle. Table 1 is comprised of the data from all experiments in which *any* response to stimulation of the sympathetic or parasympathetic nerves was obtained. In a number of these experiments it was possible to record in one and the same preparation responses to sympathetic as well as parasympathetic stimulation. These data are presented in Table 2. It contains all possible combinations of the directions in which the muscle may move.

With the recording technique employed, it was not possible to obtain in each experiment directionally opposite movements from sympathetic and parasympathetic stimulation. The results from parasympathetic excitation are considered first because they were always uniform.

1. Parasympathetic stimulation (ciliary ganglion)

a. *Recording in a radial direction* (fig. 1-A)

Stimulation of the parasympathetic ciliary ganglion caused the ciliary muscle to move invariably outward in a radial direction as demonstrated by the downward swing of the tracing in Figure 2-A. This movement was always accompanied by a symmetrical pupillary constriction. The direction of movement did not depend upon the location of attach-

TABLE 1*

CILIARY MUSCLE RESPONSES TO STIMULATION OF SYMPATHETIC AND PARASYMPATHETIC NERVES

Recording Direction	Number of Experiments			
	(+) Sympathetic Stimulation	(−) Sympathetic Stimulation	(+) Parasympathetic Stimulation	(−) Parasympathetic Stimulation
Radial	12	13	0	32
	N.S.		P<0.01	
Tangentially forward	19	4	17	4
	P<0.01		P<0.01	
Tangentially backward	11	9	9	13
	N.S.		N.S.	

* Increased tension on plate pin of transducer registered as upward deflection of recording pen (symbolized by (+) sign). Decreased tension on plate pin of transducer registered as downward deflection of recording pen (symbolized by (−) sign).

ment of the loop on the ciliary muscle within the limits of 2 to 10 mm. from the limbus.

b. *Tangentially forward recording* (fig. 1-B)

When records were taken in the forward direction tangentially to the muscle, the tension on the pin of the transducer tube increased in a significant number of cases as demonstrated by the upward swing of the tracing in Figure 2-B. The increased tension could be caused by two factors: (1) a vector of the radially outward movement, (2) a backward movement. Either or both of these factors would bring about an increase in tension and so would a combination of the two.

c. *Tangentially backward recording* (fig. 1-C)

In order to determine whether or not there was a backward movement in addition to the outward movement, records were taken tangentially to the muscle in the backward direction. These records showed that in half the cases there was a decrease in tension as depicted in Figure 2-C, and in half the cases an increase in tension.

In summary, the principal movement of the ciliary muscle upon parasympathetic stimulation was radially outward. In half of the cases the outward movement was accompanied by a backward component.

2. SYMPATHETIC STIMULATION

a. *Recording in a radial direction* (fig. 1-A)

Stimulation of one of the long ciliary nerves resulted in a radially inward movement of the ciliary muscle in half of the cases as illustrated by the upswing of the tracing in Figure 3-A. In the other half of

TABLE 2*

DIRECTION OF MUSCLE MOVEMENT UPON SYMPATHETIC EXCITATION COMPARED WITH MOVEMENT OF SAME MUSCLE UPON PARASYMPATHETIC EXCITATION

Recording direction	Number of Experiments			
	(+) Sympathetic (−) Parasympathetic	(−) Sympathetic (+) Parasympathetic	(+) Sympathetic (+) Parasympathetic	(−) Sympathetic (−) Parasympathetic
Radial	12	0	0	13
Tangentially forward	1	0	16	3
Tangentially backward	4	0	6	6

* See Table 1 for explanation of symbols.

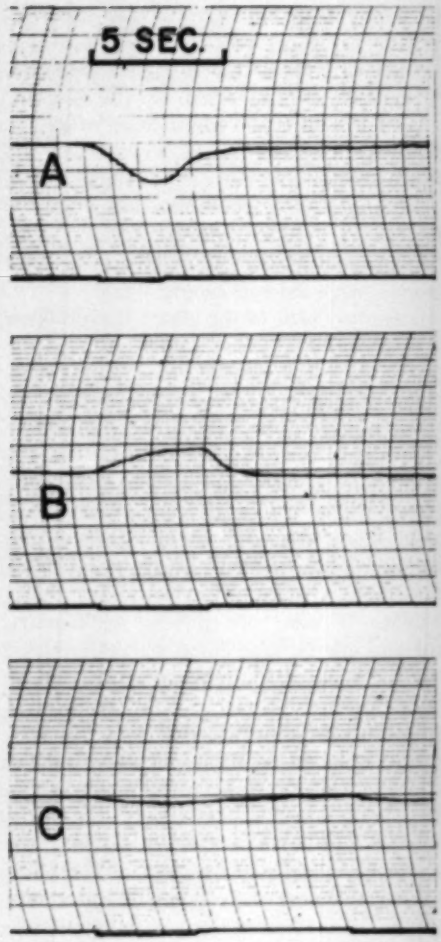

Fig. 2 (Melton, Purnell and Brecher). Response of ciliary muscle to parasympathetic stimulation. Records taken in three directions from a single point on the ciliary muscle. (A) Recording in radial direction; decrease in tension = 45 mg. (B) Recording in tangentially forward direction; increase in tension = 24 mg. (C) Recording in tangentially backward direction; decrease in tension = 10 mg. (Upper tracing: myogram. Lower tracing: stimulus.)

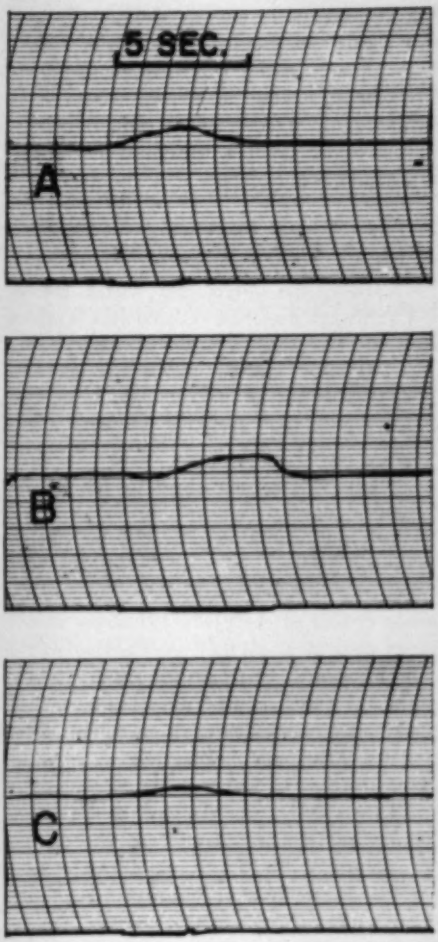

Fig. 3 (Melton, Purnell and Brecher). Response of ciliary muscle to sympathetic stimulation. Records taken in three directions from a single point on the ciliary muscle. (A) Recording in radial direction; increase in tension = 25 mg. (B) Recording in tangentially forward direction; increase in tension = 20 mg. (C) Recording in tangentially backward direction; increase in tension = 10 mg. (Tracings as in Figure 2.)

the cases the movement was the same as that which occurred upon parasympathetic excitation. Sympathetic stimulation was accompanied by a pupillary dilatation. This was always asymmetrical in the meridian of the stimulated nerve fibers. In most cases the dilatation was ill-defined and preceded by a brief, slight constriction.

b. *Tangentially forward recording* (fig. 1-B)

When records were taken tangentially to the muscle in the forward direction there

was an increase in tension on the transducer in a significant number of cases, as shown by the upward swing of the tracing in Figure 3-B. This increase in tension could have resulted from a vector of the radially inward movement or from a backward movement or a combination of both movements.

c. *Tangentially backward recording* (fig. 1-C)

Records taken tangentially to the muscle in a backward direction showed that in about half the cases there was an increase in tension (fig. 3-C) and in the rest a decrease upon stimulation of the sympathetic nerves. However, without exception, when the radial record showed an increase in tension both tangential records also showed a tension increase.

When the muscle was excited by sympathetic as well as by parasympathetic impulses, the direction of the response was opposite in half of the cases. When the movements were identical they were always of the type characteristic of parasympathetic stimulation. In no case has stimulation of the ciliary ganglion yielded a response similar to that obtained upon sympathetic stimulation. In those cases in which the responses were in opposite directions radially, simultaneous stimulation of both sets of nerves yielded no response.

In summary, upon sympathetic stimulation a radially inward movement of the ciliary muscle was demonstrated.

Discussion

The experiments reported here demonstrate that the ciliary muscle shows a definite movement in response to direct stimulation of the sympathetic nerves. This movement can only be caused by a contraction of the ciliary muscle itself. It should be emphasized that, under the strictly controlled conditions of the enucleated eye, impacts from extraocular muscle movements, deformation of the eyeball from intraocular pressure changes, and variations in shape and size of the ciliary body through vascular engorgement or depletion were ruled out. It is now possible to explain, at least in part, the effect of sympathetic excitation on the dioptric power of the lens as a direct result of ciliary muscle contraction. This direct muscular action does not rule out the possibility of an additional mechanism in the intact eye: a decrease in volume of the ciliary body through constriction of the ciliary vasculature as suggested by Fleming.[1]

The movement of the ciliary muscle upon sympathetic stimulation was opposite in direction to that obtained upon parasympathetic stimulation. These opposing movements cancelled each other in all cases when sympathetic and parasympathetic nerves were simultaneously excited. Whereas the response of the ciliary muscle to parasympathetic stimulation was always uniform, the non-uniform reaction upon sympathetic stimulation requires an explanation.

Since it was very difficult to identify anatomically the long ciliary nerves, we used the dilatation of the pupil as a criterion of sympathetic excitation in order to identify the sympathetic nerves. However, this procedure is based on the assumption that the same sympathetic nerve fibers which supply the *dilator pupillae* also supply the ciliary muscle. This is not necessarily so. Since the sympathetic and parasympathetic fibers are intertwined near their point of entry into the eye (Christensen[11]), it is quite possible that predominantly sympathetic fibers leading to the iris, but predominantly parasympathetic fibers leading to the ciliary muscle, were stimulated. In fact, in such a randomly arranged system, this result is to be expected. This would explain the fact that in half of the cases of sympathetic stimulation as manifested by pupillary dilatation, the response of the ciliary muscle was a radially outward movement, just as that obtained by excitation of the parasympathetic ciliary ganglion. This explanation, based on an unequal distribution of autonomic fibers to the ciliary and iris muscle, is also supported by

the histologic findings of Warwick.[12] He has shown that only about three percent of the parasympathetic fibers whose cell bodies lie in the ciliary ganglion go to the iris. The rest are presumed to go to the ciliary muscle.

We interpret the inward and outward movements of the exposed ciliary muscle as those which produce tension changes on the zonule. In the preparation used in this study, the fibrous connections anchoring the ciliary muscle and choroid to the sclera were missing and were replaced by the pin of the transducer tube. As recorded through a scleral window, the inward pull upon sympathetic stimulation results from shortening of the radial muscle fibers and causes increased tension on the zonule and flattening of the lens. The opposite situation would obtain upon parasympathetic stimulation. The outward movement of the muscle represents lengthening of radial muscle fibers and consequent decreased tension on the zonule. This interpretation fits with the lens changes reported by Olmsted and his collaborators.[4-7]

Implicit in this interpretation of the movement of the ciliary muscle of the cat is the idea that the radial muscle layer is the principal effector of accommodation in this species. Contraction of it is caused by sympathetic nerve impulses and results in lens flattening; relaxation of it is caused by parasympathetic nerve impulses and results in lens thickening. This relaxation may be due to inhibition of autonomous tone of the radial layer, a phenomenon well known in smooth muscle. The backward movement of the muscle can be interpreted as a participation of the meridional layer in producing near accommodation. Such a movement would tend to relax tension on the forward-directed zonular fibers. The assignment of prime responsibility for accommodation to the radial layer of the cat is consistent with Walls'[13] statement that the orders below primates have very little, if any, circular muscle layer.

Summary

In strictly controlled experiments, the question was resolved whether or not the ciliary muscle fibers contract upon sympathetic nerve stimulation. In order to exclude extraocular movements, intraocular pressure changes, the sympathetic (long ciliary) as well as parasympathetic (short ciliary and ciliary ganglion) nerves were directly stimulated in the excised cat eye. Stimulation of parasympathetics or sympathetics produced marked movements of the ciliary muscle as recorded through scleral windows. The problems of eliciting a sympathetic response is discussed in detail. Upon parasympathetic stimulation the principal movement was in a radially outward direction, and upon sympathetic stimulation, in a radially inward direction. The radially inward movement of the ciliary muscle to sympathetic stimulation is caused by a contraction of the radial muscle fibers in the ciliary body. The radially outward movement upon parasympathetic stimulation may be due to an inhibition of autonomous tone of the radial fibers. The effect of sympathetic excitation on distance accommodation can be explained, in part, as a direct action of the sympathetic nerves on the radial muscle fibers of the ciliary body.

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Discussion

Dr. Peter C. Kronfeld (Chicago, Illinois): I would call this a neat piece of work, neat in design and neat in execution.

We have been shown a set of observations supporting the concept that the radial muscle fibers in the ciliary body of the cat are under the direct and positive control of the sympathetic, and that the activity of these fibers constitutes a major factor in the process of accommodation.

At first glance the evidence shown may not seem very consistent. I believe the essayists are justified in attributing some of the inconsistencies to impurity and indefiniteness of the long ciliary nerves. I ran into that difficulty about 15 years ago, when I became interested in stimulation of the sympathetic behind the globe. A number of times I observed that stimulation of a nerve which seemed to fulfill the anatomic requirements for a long ciliary nerve, caused no pupillary response. Apparently I was dealing with a mixed motor nerve or a purely sensory nerve.

There is one other point that may account for some of the inconsistencies encountered by the essayists. The part of the muscle which they connected with the recording instrument is close to the surface of the muscle and pretty far forward, that is, close to the origin of the muscle. If the muscle is to function effectively, the origin should not move at all. The ciliary muscle in the cat may be constructed in such a way that the outermost layers of the ciliary body, particularly in their anterior portion, do not move very much. Such a concept could explain both the slight inward and the slight outward movement observed by the essayists.

I am satisfied that the essayists have proven their main point, that is, the antagonistic nature of the responses to parasympathetic stimulation, on the one hand, and to sympathetic stimulation on the other. The question might be asked whether the results presented today tally with those presented here by Sachs of Detroit in 1942. In those experiments electric stimuli were applied directly to the exposed outer surface of the ciliary muscle. Chalk marks had to serve as indicators of any movements occurring during the stimulation.

Dr. Sachs' technique was not suitable for the demonstration af a radial component, but did show clearly a backward movement of the anterior part of the ciliary muscle and a forward movement of the posterior portion of the ciliary muscle. The present essayists have confirmed the former but not the latter component of ciliary muscle movement. The difference in the type of stimulation may account for the difference in response.

This study adds very materially to our knowledge of the mechanism of accommodation.

Dr. C. E. Melton (in closing): I would like not only to express our gratitude, but to say that I think we are extremely fortunate in having Dr. Kronfeld discuss our work and give us the benefit of his wide experience in this field.

He mentioned the large number of failures which he encountered in attempting to stimulate the sympathetic nerves. I would like to emphasize that I have not reported our failures, which were numerous.

He mentioned cervical-sympathetic stimulation. We have tried many times to demonstrate this effect in vivo; and we find that upon stimulation of the cervical sympathetics, there are changes in tension on the transducer; however, these movements cannot be separated from extraocular muscle movements and vascular changes.

I did not mention the meridional movement. We feel that this is a participation of von Bruecke's muscle in effecting near accommodation, that this movement tends to relax or slacken the forward-directed zonular fibers.

We are very much aware of the excellent work of Dr. Sachs. We feel that we in no way stand in contradiction to this work, and that we have merely added another direction of recording.

SPONTANEOUS AND RADIATION-INDUCED IRIS ATROPHY IN MICE*

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Knoxville, Tennessee

Iris atrophy was noted in LAF_1 mice exposed to ionizing radiations from an experimental nuclear detonation during observation of the animals for cataract development (Furth et al., 1954). The following is a preliminary report of the clinical and morphologic features of this disease.

Methods

Male and female mice of the LAF_1 strain (first-generation hybrid of C_{57} L ♀ X A ♂) exposed to acute whole-body radiation at six to 12 weeks of age were examined periodically throughout life with a Zeiss Opton slitlamp, at a magnification of 16 times or greater. The animals under observation numbered more than a thousand and were grouped as follows: (1) Nonirradiated mice; (2) mice of the same strain exposed to 25-500 r of X rays; and (3) several subgroups exposed to various graded doses of ionizing radiations from an experimental nuclear detonation (Furth, et al., 1954). The eyes of the animals were examined, with the pupil undilated, and the condition of the iris was recorded for each mouse, according to the grading system described hereafter.

Clinical and Histologic appearance of the iris

The normal iris (grade 0) of the LAF_1 mouse is shown in Figure 1. The anterior surface appeared smooth, resembling velvet. In most instances small floccules were seen at the pupillary margin. The earliest degree of atrophy (grade 1) manifested itself as a faint, linear, radial streaking of the iris (fig. 2); the streaks were black, distinct from the brown color of the normal iris. There was also a slight roughening of the anterior iris

Fig. 1 (Benedict, Christenberry, and Upton). A sketch of the normal iris of the LAF_1 mouse, exhibiting small floccules at the pupillary margin.

stroma, with disappearance of the pupillary floccules. The radial ridges around the pupil were accentuated, and the pigment gathered

Fig. 2 (Benedict, Christenberry, and Upton). A drawing of an iris manifesting grade-1 atrophy. Pupillary floccules are no longer present, and there is accentuation of the radial ridges around the pupils.

* From the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee. This work was performed under USAEC Contract No. W-7405-eng.-26.

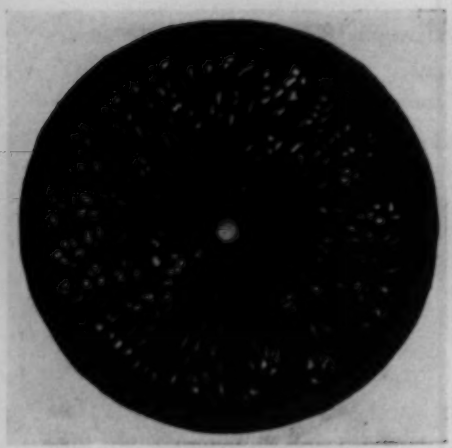

Fig. 3 (Benedict, Christenberry, and Upton). A sketch of an iris with grade-2 atrophy, exhibiting numerous small holes.

into granular masses on the iris surface. By retroillumination, the iris had a lacy, mottled appearance which helped considerably in grading the early changes, and it usually contained a small perforation.

Eyes that showed more than one hole were placed in grade 2 (fig. 3); at this stage the anterior surface of the iris appeared rougher than in grade 1. In addition, there was some loss of the anterior stroma, without much involvement of the underlying neuroepithelial layer. The lacy network noted with grade 1 involvement was more marked (fig. 4), and the perforations were often numerous and relatively large, owing to coalescence

Fig. 4 (Benedict, Christenberry, and Upton). An iris with grade-2 atrophy, as photographed through the slitlamp, manifesting lacy perforations in the midperiphery.

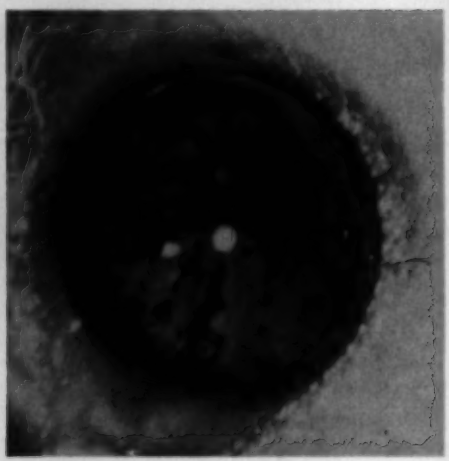

Fig. 5 (Benedict, Christenberry, and Upton). An iris, as photographed through the slitlamp, with grade-3 atrophy, exhibiting loss of one-half to three-fourths of its stroma.

Fig. 6 (Benedict, Christenberry, and Upton). A drawing of an iris with grade-3 atrophy, showing single strands of iris adherent to the anterior lens surface.

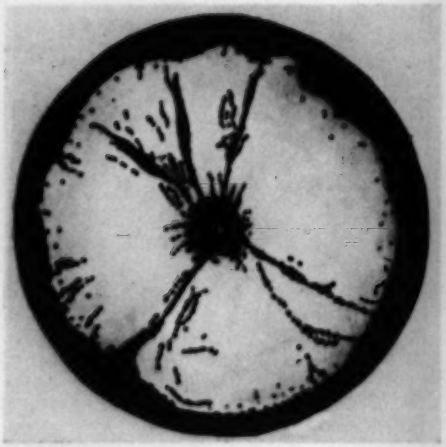

Fig. 7 (Benedict, Christenberry, and Upton). A sketch of an iris with grade-4 atrophy, exhibiting loss of virtually all of its stroma.

of two or more holes. The midperiphery of the iris was the region most commonly involved, the zone around the sphincter remaining relatively intact. In advanced stages of grade 2 involvement there were clumps of pigment in the chamber angle, filling the space between the cornea and peripheral iris.

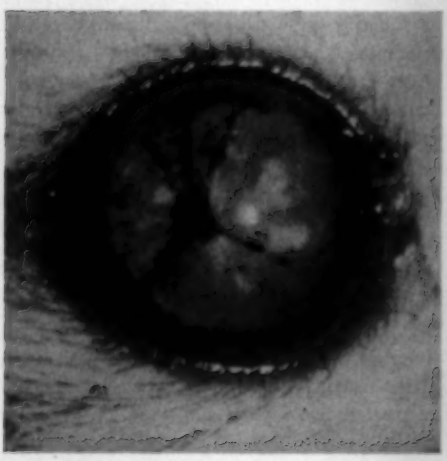

Fig. 8 (Benedict, Christenberry and Upton). An iris, as photographed through the slitlamp, showing grade-4 atrophy; only a few flecks of isolated iris tissue remain.

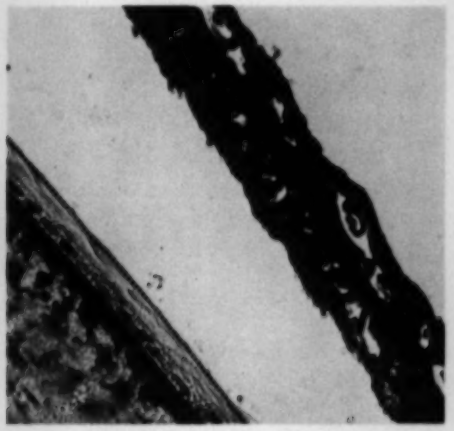

Fig. 9 (Benedict, Christenberry, and Upton). A histologic section of a normal iris, exhibiting the anterior and posterior layers; the blood vessels of the former are clearly defined. (Hematoxylin-eosin, ×400.)

In grade 3 the iris showed loss of one-half to three-fourths of its stroma (fig. 5). Clumping of pigment in the anterior chamber angle was more obvious. There were usually single strands of iris tissue running

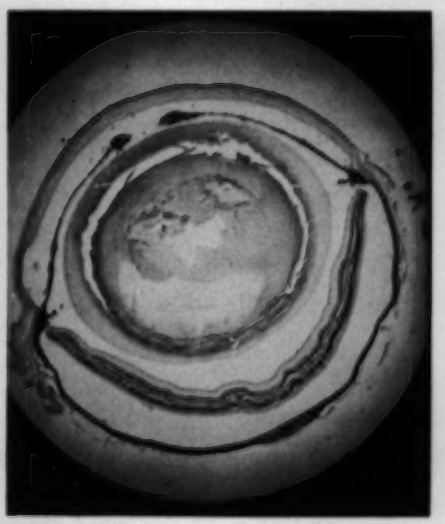

Fig. 10 (Benedict, Christenberry and Upton). A section of an eye with an atrophic iris which exhibits marked hypertrophy of the sphincter pupillae. (Hematoxylin-eosin, ×25.)

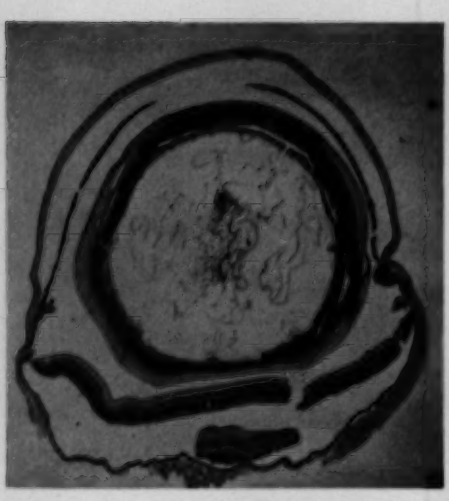

Fig. 11 (Benedict, Christenberry, and Upton). A section of a normal eye, showing the sphincter pupillae of normal size. (Hematoxylin-eosin, ×25.)

radially across the anterior lens surface (fig. 6), and the iris appeared adherent to the lens capsule. The pupil was usually small, with pigment cells clumped about it, giving the sphincter a hypertrophic appearance.

In grade 4 there was absence of nearly all of the iris (fig. 7); the stump of the iris was seen deep in the chamber angle, and a few flecks of isolated pigment cells were sometimes seen on the anterior lens surface (fig. 8). The chamber angle was completely occluded in some eyes, while in others synechiae were no more numerous than in grade-2 atrophy. The sphincter pupillae was largely gone; any remnants of it, however, were not much displaced.

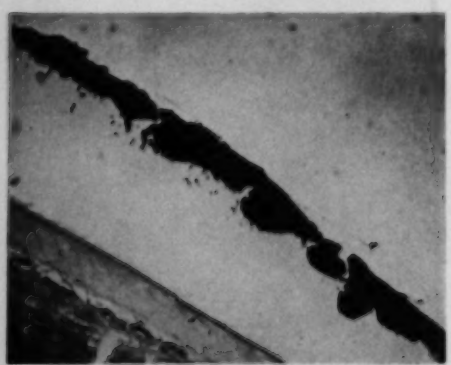

Fig. 12 (Benedict, Christenberry and Upton). A section of an atrophic iris exhibiting a loss of stroma and vessels and slight enlargement of the chromatophores. (Hematoxylin-eosin, ×400.)

Fig. 13 (Benedict, Christenberry, and Upton). A section of an eye with grade-3 iris atrophy; there is marked discontinuity of the iris because of loss of stroma. (Hematoxylin-eosin, ×25.)

None of the eyes exhibited signs of iritis; an aqueous ray was not present, and keratitic precipitates were not seen. In the eyes with grade-4 atrophy, residual anterior chamber hemorrhages were sometimes observed. In these eyes, the chamber was extremely deep and the cornea diffusely hazy. The etiology of the hemorrhage was obscure; the blood vessels of the iris could not be seen with the slitlamp in these eyes, probably because of their heavy pigmentation.

Histologically, the significant observed changes were limited to the iris, the anterior chamber angle, the ciliary body, and, to a lesser extent, the choroid. The iris in the normal mouse is a tissue with two distinct layers, neuroepithelial and mesenchymal, blood vessels being numerous and easily seen in the latter, even in unbleached specimens

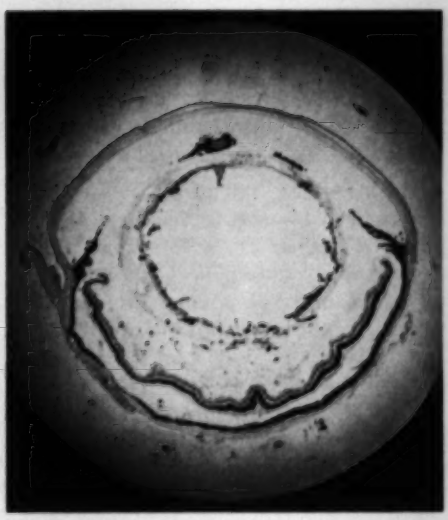

Fig. 14 (Benedict, Christenberry, and Upton). A section of an eye with grade-4 iris atrophy; the loss of iris tissue is even more marked than that shown in the preceding figure. (Hematoxylin-eosin, ×25.)

(fig. 9); however, in early essential atrophy there was disorganization of the iris, with loss of some of the mesodermal tissue. The sphincter pupillae muscle was hypertrophic (fig. 10) and often reached several times its normal size (fig. 11).

The cells in the neuroepithelial layer became larger and rounder; concurrently, their pigment became more conspicuous, as an accumulation of many tiny, round granules (fig. 12). Some such cells appeared to have ruptured, dispersing their pigment granules. In the more advanced stages, the cells seemed to be separated from one another; the iris became one cell layer in thickness, and its vascular network disappeared (fig. 12).

Histologically, in grade 3 atrophy (fig. 13) the iris revealed the large gaps seen clinically, and in grade 4 (fig. 14) the discontinuity of the iris was even more marked. In the anterior chamber angle, free, large cells containing pigment granules were observed, located chiefly between the iris root and the trabeculum (fig. 15); Schlemm's canal was

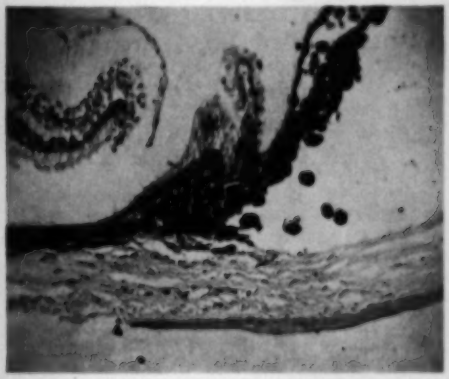

Fig. 15 (Benedict, Christenberry, and Upton). A section demonstrating hypertrophy of the ciliary body and processes, with accumulation of free chromatophores in the angle of the anterior chamber. (Hematoxylin-eosin, ×150.)

identified in most of the sections, and pigment cells or pigment granules were not observed in it. At no time was the chamber angle occluded by fibrosis.

The ciliary body and ciliary processes were somewhat hypertrophic in the most advanced cases. The choroid, in the eyes of mice with marked iris atrophy, also took part in the pigment disturbance (fig. 16). The large cells containing pigment granules in the choroid were indistinguishable from those in the iris; however, since the choroid

Fig. 16 (Benedict, Christenberry, and Upton). A section of the choroid in an eye with grade-4 iris atrophy. The chromatophores exhibit enlargement, and there is dispersion of pigment granules. (Hematoxylin-eosin, ×400.)

TABLE 1

IRIS ATROPHY IN RELATION TO DOSE OF X-RADIATION

Dose* (r)	Total No. of Mice Examined†	Severity of Iris Atrophy	
		Grade 1	Grade 2
		No. of Mice	
500	14	1	13
400	18	4	14
300	16	6	10
200	18	10	8
100	20	10	10
50	17	12	5
25	18	16	2
0	21	14	7

* 230-kvp X rays, 15 ma; TSD, 100 cm; filter, 1 mm of Cu plus 1 mm of Al; HVL, 1.93 mm of Cu; dose rate in air, 14 r/min.

† The mice were irradiated at 6–9 weeks of age and were 22–23 months old at the time of the above analysis. The nonirradiated controls were obtained from the same colony as the irradiated mice (U. S. Naval Radiological Defense Laboratory) but received later; however, in the above analysis all mice were matched with respect to age.

was compressed between the sclera and retina, the individual cells were not so clearly seen as in the iris.

None of the sections exhibited inflammatory cells or signs of previous inflammation.

Relation of iris atrophy to irradiation

More than 1,000-normal and irradiated LAF_1 mice have been examined with the slitlamp; all have shown iris atrophy on aging. The involvement of the iris was bilateral and both sexes were affected similarly. Animals exposed to ionizing radiation exhibited an earlier onset, more rapid progression, and a more advanced final degree of atrophy than nonirradiated controls, in proportion to the dose of radiation received (table 1); in aging controls the atrophy was first detectable at about 17 months of age and progressed to grade-2 severity in senility (24 to 40 months), whereas in heavily irradiated mice it appeared as early as the 14th month, often progressing to grade 3 or 4 before death.

Discussion

Although this disease, which appears to be an hereditary trait of the LAF_1 mouse, is exaggerated by irradiation, it has not been observed in other irradiated animals, and its significance is uncertain. The pathogenesis of the iris atrophy is unknown. Similarly, it is not clear whether irradiation exacerbates this condition by local damage or by systemic injury; it is conceivable that the mechanism is related to acceleration of the aging process, which is also proportional to the dose of radiation received (Upton and Furth, 1954). The blood vessels of the iris, which are not easily visualized through the slitlamp, are being studied by other methods to evaluate their role in the pathogenesis of this condition.

Summary

Atrophy of the iris was observed to occur in all aging LAF_1 mice.

The atrophy, which resembled in certain respects essential iris atrophy in the human eye, appeared sooner and progressed to greater severity in irradiated mice than in controls.

The morphologic features of the disease are described.

Shelbourne Towers.

Acknowledgement

The authors are grateful to Mr. W. D. Gude, Mrs. E. S. Ledford, and Mrs. F. F. Wolff for technical assistance. It is also a pleasure to express our indebtedness to Dr. V. P. Bond et al., of the U. S. Naval Radiological Defense Laboratory, from whom many X-irradiated LAF_1 mice were obtained.

References

Furth, J., Upton, A. C., Christenberry, K. W., Benedict, W. H., and Moshman, J.: Some late effects in mice of ionizing radiation from an experimental nuclear detonation. Radiology, **63**:562-570, 1954.

Upton, A. C., Furth, J., and Christenberry, K. W.: Late effects of thermal neutron irradiation in mice. Cancer Research, **14**:682-690, 1954.

Discussion

Dr. Parker Heath (Sullivan Harbor, Maine): Spontaneous atrophies of the iris, with hyperplastic ciliary body and choroidal changes of LAF_1 strain of mice, are advanced and augmented by exposure to ionizing radiation. The spontaneous and the radiation-induced lesions are equally interesting. The latter may be summarized, strictly from the published data of the author, as the reduction of longevity, graying, blood dyscrasias (ovarian, pituitary, harderian, mammarian-gland, and others), tumors, nephrosclerosis, and ocular lesions.

The ocular lesions, briefly stated, are uveal atrophies and hyperplasias and cataracts.

Histopathology, as found in the irises, includes disorganizations and losses of tissue, including the blood vessels and pupil-border floccules; hyperplasias of sphincter muscle; enlarged, questionably proliferated chromatoblasts, and scattered pigment.

The iris-corneal angles of the mice apparently were not altered much except during late and severe stages, when the anterior chambers were deepened.

Ciliary bodies presented hypertrophy and possibly hyperplasias during late stages.

Our special interest, of course, is to transport or translate the mouse pathology to man. An attack on this problem may be made by considering differences between the mouse disease and the human. The highly complicated, diverse, debatable, and interesting radiation effects apparently are augmented and sped up by what appears to be an abiotrophic process. Certainly, new factors are also introduced to the genetic disease. Therefore, because of the radiation complexities, it is probably more profitable to attempt to consider the spontaneous atrophies of mouse compared with those of man. These considerations are made strictly from the papers of Benedict et al., and are not the result of examination of specimens.

A listing of some of the differences between mouse LAF_1 strain atrophies and man is as follows:

1. Clear-cut heredity in mouse, not true with man.

2. Sex involvement about equal in mouse, quite unlike man, where the disease is heavily weighted toward the female side.

3. Uniformity of locus; that is, the pupils are not markedly displaced.

You noticed, in the essayists' illustrations, the pupils were centrally placed, even in extreme degrees of atrophy. In man, the pupillary space or ring migrates to one side almost always.

4. Hyperplasias affecting sphincter, ciliary body, and choroid are quite unlike those changes in man.

5. Absence of or no emphasis on glaucoma is unusual in the mouse; whereas this disease is common in man.

6. Absence of glass-membrane formations are common in man, 50 percent in our experience.

7. Grayness (that is, joint depigmentation of coat and iris atrophy) is not characteristic of man but is of the mouse.

8. Choroidal melanoblastic proliferations in the mouse are unknown to man in my experience.

9. The time of appearance and the more regularity of this appearance are true with the mouse but not with man.

No doubt, other differences could be elaborated, but those listed above supply some of the hurdles which the essayists, and so forth, all must surmount before the disease of mouse and man can be called one and the same.

The crux of the problem is related to what takes place in the blood vessels of the iris and the ciliary body. After due regard to the morphology and distribution of the mouse blood vessels, are mass occlusions demonstrable? Are these distributed by blood systems to zones of atrophy?

The answers to these and many other questions will no doubt be supplied by future studies; we shall look forward to additional reports. At this time, the material presented by the authors is of great interest within many fields of medicine and also genetics.

Dr. William F. Hughes, Jr. (Chicago, Illinois): Dr. Benedict made one point, that the changes in the iris might possibly be on a systemic or a local basis. Dr. McDonald and I, in studying radiation cataracts and beta irradiation, found that such iris atrophy occurred with localized radiation applied to the limbus. The exact dosage required to produce that, I have forgotten temporarily.

Dr. W. H. Benedict (in closing): I wish to thank Dr. Heath and Dr. Hughes for their comments and discussion of the paper.

After listening to Dr. Falls the last two or three days, I began to wonder if maybe these animals do not resemble the animal counterpart of the Axenfeld syndrome because, later in the stage, they develop some corneal opacity. Whether this is due to injury when the mice run into each other or whether they develop it spontaneously is a little difficult to say at the present time.

I neglected to show, in my final table, what happened to the controls. In the published version of this paper, there will be a much better, more elaborate chart, which you can use for comparison.

These animal changes take approximately a year and a half to two years before they really begin to show something exciting and good to look at. Consequently, it will be another several years before we have more detailed information as to pathogenesis.

We have attempted to trace the genetics of these animals; but we have, at the present time, been able to get a sufficient number of only the A half of the parents; the other half of the parents do not breed very well. At the present time, there is nothing showing up in the A's we have followed.

THE EFFECT OF BETA RADIATION ON CORNEAL HEALING*

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Chicago, Illinois

The possible inhibition of healing of corneal wounds by beta radiation has been noted clinically in two patients who developed filtering blebs following cataract extraction,[1] and in a few patients who had received beta radiation for corneal vascularization prior to corneal transplantation at the Illinois Eye and Ear Infirmary. Such delay in the healing of wounds has been reported following irradiation in other parts of the body; for example, formation of spontaneous fistulas into the bladder which were difficult to close following radium treatment for carcinoma of the cervix,[2,3] difficulty of primary closure and danger of late breakdown of irradiated tissue following mastectomy,[4] and the relative difficulty of grafting skin in irradiated areas. Although there are some experimental reports suggesting that very small doses of radiation can stimulate healing of skin wounds in animals,[5-8] it is generally agreed that larger doses such as 1,000 r of X rays or 100 mg. hr. of radium will inhibit healing of skin incisions.[8-11]

The experimental work on corneal wound healing following exposure to Grenz rays,[12] ultraviolet and X rays,[13] and beta particles[14] has been limited to the effect on the healing of epithelial defects alone. With threshold doses, there is a temporary inhibition of mitosis followed by a rebound excess of mitotic figures. With larger doses, nuclear fragmentation of the corneal epithelium occurs.

It is the purpose of the present study to determine the effects of beta radiation on the healing of wounds in the corneal stroma of rabbits.

MATERIALS AND METHODS

The source of beta particles used in these experiments was the Strontium^{90}-Yttrium^{90} applicator manufactured by Tracerlab. It had a physically calibrated output of 104 rep./sec., and an active face of 5.0 mm. The dose required to produce a permanent corneal nebula was 25,000 rep. Mongrel, pigmented rabbits were used, approximately two to three months of age and weighing from four to five lb.

Experiment I determined the minimum and optimum dose necessary to inhibit corneal wound healing. After anesthesia with intravenous sodium phenobarbital and topical pontocaine, one eye of each rabbit was irradiated in the center of the cornea with doses of 1,250, 2,500, 5,000, 10,000, and 20,000 rep. (10 rabbits for each dose). Immediately afterward, a five-mm. incision penetrating into the anterior chamber was made in the center of the cornea with a cataract knife. Atropine and sulfacetamide ointment were then applied. An identical procedure was done on the opposite eye of each rabbit except that no irradiation was given. Slitlamp examinations, photographs, and histologic sections were made at regular intervals up to six months.

In Experiment II, 5,000 rep. were given at varying intervals prior to surgery. Four animals were used for each time interval; three days, one week, two weeks, one month, and three months lapsing between the radiation and the surgery. The opposite eyes of these animals were treated similarly without any radiation. Histologic sections were made two weeks after surgery on representative eyes. In addition, six eyes of three rabbits which had received a dose of 27,000 rep. about two years prior to surgery were followed similarly.

Experiment III. In 10 rabbits of Experiment III, a five-mm. limbal incision without a flap was made into the anterior chamber in an area previously exposed to 5,000 rep. A similar incision was made in the opposite

* From the Department of Ophthalmology, University of Illinois College of Medicine.

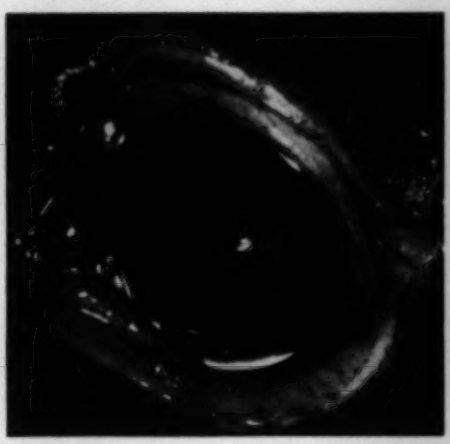

Fig. 1A (McDonald and Wilder). 5,000 rep. beta given just prior to surgery; one week postoperative. Note wound gaping and deficiency of wound plug.

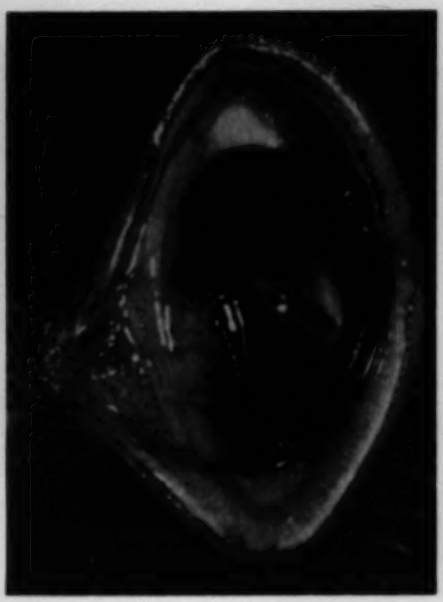

Fig. 1B (McDonald and Wilder). Control eye. Note solid plug between wound edges.

eyes of these animals without previous radiation.

Results

Experiment I

Irradiation immediately before an incision was made through the center of the cornea resulted in a gross inhibition of healing in all doses except for the smallest dose of 1,250 rep. where the results were equivocal. This gaping of the incision was manifest

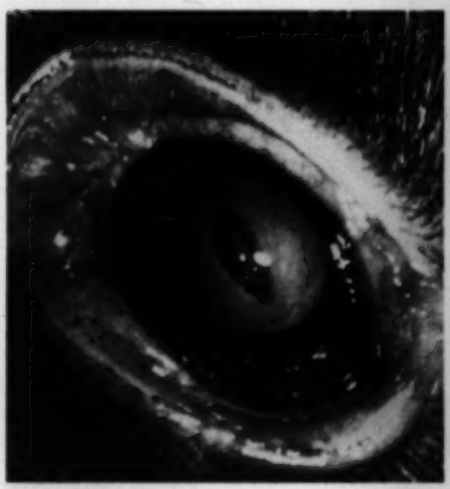

Fig. 2A (McDonald and Wilder). Same eye as in Figure 1A two weeks postoperative. The continued wound weakness is apparent.

Fig. 2B (McDonald and Wilder). Control eye. The eye is already well healed by dense tissue.

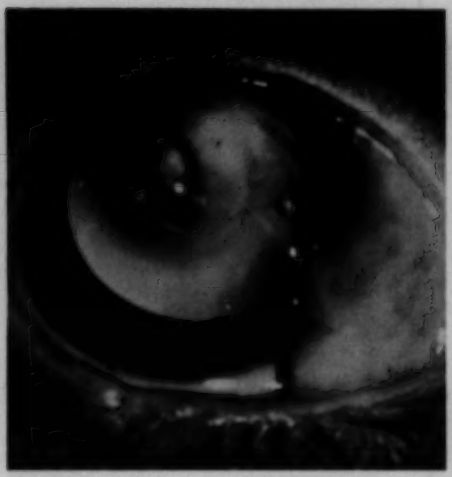

Fig. 3A (McDonald and Wilder). Same eye as in Figure 1A one month after radiation and surgery. Thin gaping wound edges are seen.

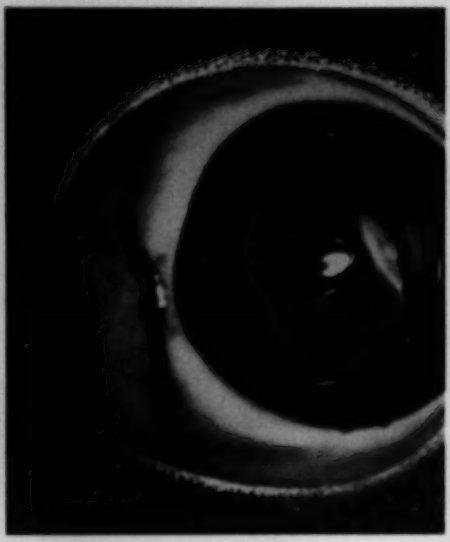

Fig. 3B (McDonald and Wilder). Control eye. The dense white scar tissue makes a firm wound.

after one week and became more pronounced two weeks following surgery in all of the rabbits studied (figs. 1 to 4). In most cases after two weeks, the irradiated eyes showed a posterior dehiscence of the wound. The irradiated wounds contained a plug which seemed less dense and in some cases appeared grossly porous as compared with the control. Corneal edema surrounded both the radiated and control lesions in the initial stages. The edema was greater in the radiated eyes in proportion to the dosage and

Fig. 4A (McDonald and Wilder). 2,500 rep. beta given just prior to surgery; two weeks postoperative. Gaping and lack of dense plug are similar to Figure 2A, which had a larger dose.

Fig. 4B (McDonald and Wilder). Control eye. Dense wound plug is seen.

Fig. 5A (McDonald and Wilder). 5,000 rep. beta just prior to surgery; six months postoperative. Edema surrounding the weakened wound edges has taken on the characteristics of scar tissue.

Fig. 5B (McDonald and Wilder). Control eye. There is the normal tendency for scar to recede.

tended to persist. In the late stages (three- to six-month reading) this edema took on the characteristics of a diffuse scar surrounding the beta-weakened wound (fig. 5). In a few cases the surrounding scar assumed the characteristic of a polycystic mass which was raised above the level of the cornea, and was associated with some vascular reaction (fig. 6).

Histology. The characteristic histologic picture of the radiated incisions seemed to be a lack of normal fibroblastic (keratoblastic) proliferation. After two days, the epithelium had grown down into the bottom of the irradiated wound (fig. 7A), and no sign of elongated fibroblastic cells was visible along the edges of the wound such as was seen in the control eye (fig. 7B). After one week the keratoblastic activity was pronounced in the control eye (fig. 8B), whereas in those eyes which were irradiated in all dosages, there was almost a total absence of

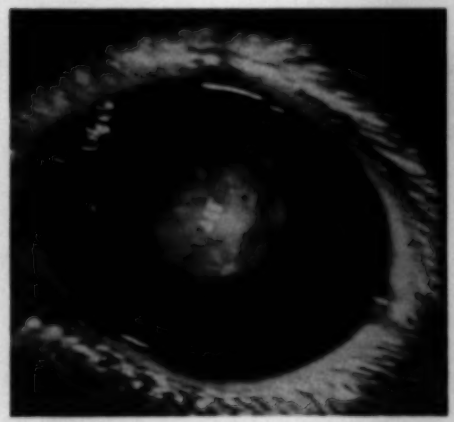

Fig. 6A (McDonald and Wilder). 5,000 rep. beta just prior to surgery; three months postoperative. This is an unusual late hypertrophic response of wound.

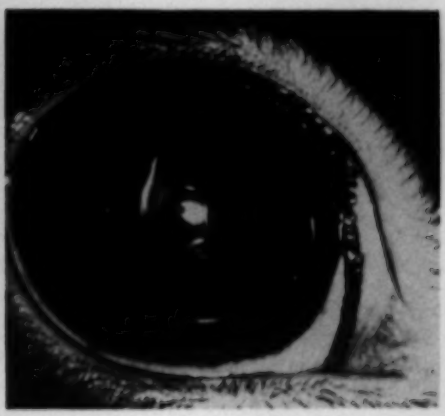

Fig. 6B (McDonald and Wilder). Control eye. Note the normal postoperative appearance.

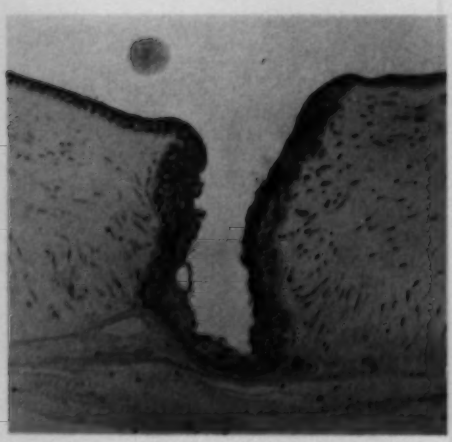

Fig. 7A (McDonald and Wilder). 5,000 rep. just prior to surgery; two days postoperative. The lack of fibroblasts and thickening of the stroma due to edema seemed to be characteristic features of the radiated wound at this stage. The ingrowth of epithelium and the fibrin deficiency was not a constant feature.

keratoblasts so that the wounds were only covered with an epithelial bridge (fig. 8A). This inhibition of keratoblasts continued into the second week (fig. 9A) and was also equally pronounced at the one month histologic sections (fig. 10A). It is to be noted

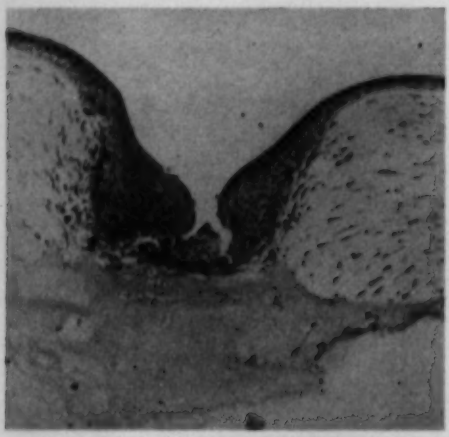

Fig. 8A (McDonald and Wilder). 5,000 rep. just prior to surgery; one week postoperative. The marked lack of fibroblasts and edematous corneal stroma is to be noted. The fibrin response doesn't seem to be affected.

Fig. 7B (McDonald and Wilder). Control eye. Note the presence of fibroblasts at the wound edges in small numbers and less edema.

that there was little effect on the proliferative ability of the epithelium or endothelium (fig. 9A) in these experiments. In the irradiated eyes, a histologic confirmation of the slitlamp findings of edema surrounding the central incision was found. Very few in-

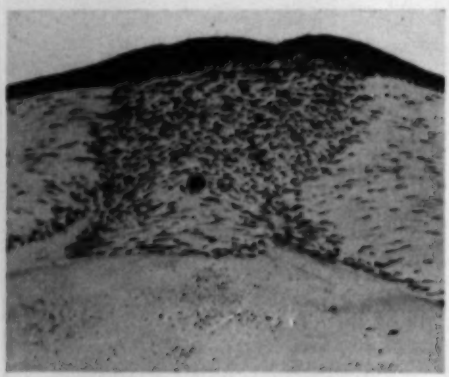

Fig. 8B (McDonald and Wilder). Control eye. Note marked fibroblastic response giving a firm wound closure.

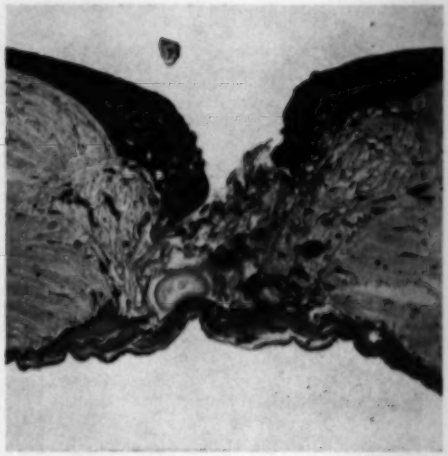

Fig. 9A (McDonald and Wilder). 5,000 rep. just prior to surgery; two weeks postoperative. Fibroblastic inhibition and stromal thickening continue. Epithelial inhibition was inconstant.

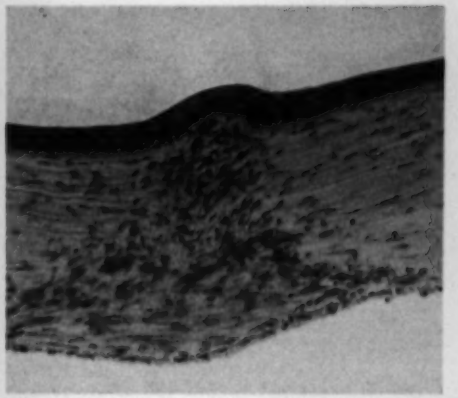

Fig. 9B (McDonald and Wilder). Control eye. This is the normal two-week appearance.

Fig. 10A (McDonald and Wilder). 5,000 rep. just prior to surgery; one month postoperative. Block of fibroblastic phase of healing mechanism has resulted in a thin bridge over the wound.

Fig. 10B (McDonald and Wilder). Control eye.

flammatory cells appeared, a picture which has been described previously as being characteristic of acute radiation burns of the cornea.[15]

The histologic appearance of the three- and six-month postoperative eyes gave a variable picture. A typical histologic finding at six months was the presence of some corneal thickening, an interruption of the horizontal lamellae at the wound site, relatively few cells and unsubstantial supporting tissue (fig. 11A). Another eye at six months had such a poorly healed wound that the edges separated at the time of section (fig. 12A).

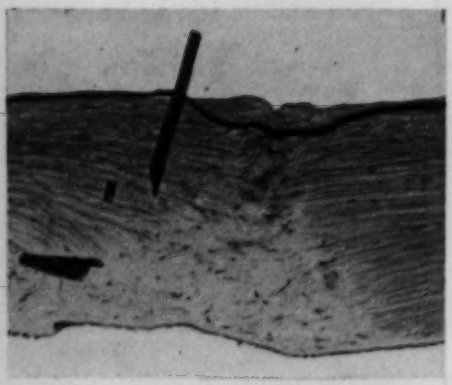

Fig. 11A (McDonald and Wilder). 5,000 rep. just prior to surgery; six months postoperative. Cornea is thickened and a few irregularly placed fibroblasts lie in the area of disrupted lamellar architecture.

Section of one of the eyes with a polycystic elevated bleb three months postoperatively revealed thickening of the cornea and irregular accumulations of epithelium, fibrous tissue, inflammatory cells, and blood vessels (fig. 13A).

Experiment II

An attempt to determine the effect of 5,000 rep. of radiation applied at different intervals before surgery showed no clinical

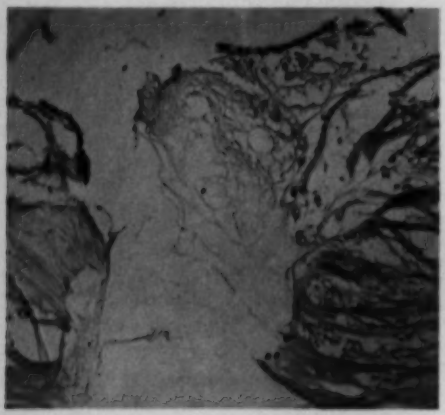

Fig. 12A (McDonald and Wilder). 5,000 rep. one month prior to surgery; six months postoperative. Stroma is irregular and sparse. Wound was so weak that it broke apart during the sectioning.

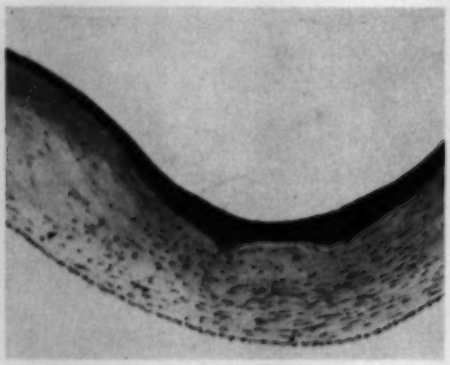

Fig. 11B (McDonald and Wilder). Control eye. Regular lamellar pattern has resumed.

difference between those eyes which were irradiated at the time of surgery as in Group I and those eyes which had been irradiated as long as three months prior to surgery (figs. 14A and B). Both showed the same inhibition of wound healing as a result of radiation in contrast to the good healing of the controls. It is to be noted here that, with this dose of radiation, the corneas looked normal to slitlamp examination prior to surgery. The six eyes mentioned which had received a large dose (27,000 to 40,000 rep.) of beta radiation about two years prior to surgery also showed a dramatic inhibition of wound healing. These corneas prior to surgery were either grossly normal in appearance or had slight scarring from the previous large dose of radiation.

Histology. The animals in Experiment II showed an inhibition of keratoblastic prolif-

Fig. 12B (McDonald and Wilder). Control eye. Note regularity of lamellar pattern.

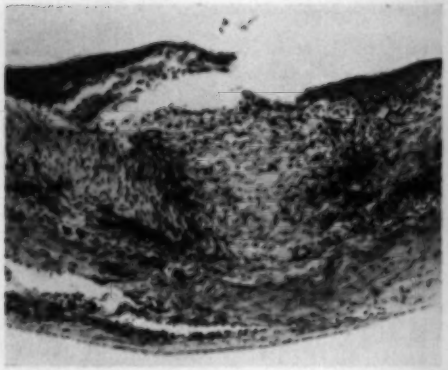

Fig. 13A (McDonald and Wilder). 5,000 rep. just prior to surgery; three months postoperative. Histology of unusual hypertropic response shown in Figure 6A. There is an irregular collection of blood vessels, chronic inflammatory cells, and fibroblasts.

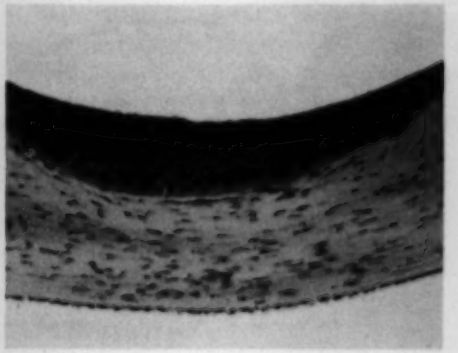

Fig. 13B (McDonald and Wilder). Control eye.

eration which could not be distinguished qualitatively or quantitatively from the histologic sections of those eyes radiated immediately prior to surgery. The same characteristic inhibition of keratoblastic activity as compared with the controls and the same acellular corneal edema surrounding the incision were present (figs. 14C and D).

Experiment III

Peripheral incisions were made about one mm. outside the limbus. Beta radiation produced gross inhibition of wound healing af-

Fig. 14A (McDonald and Wilder). 5,000 rep. three months before surgery; one month postoperative. Inhibition of healing is the same as if given just prior to surgery. This similarity between those radiated just prior to surgery and those radiated months before surgery was noted in all postoperative observations.

Fig. 14B (McDonald and Wilder). Control eye.

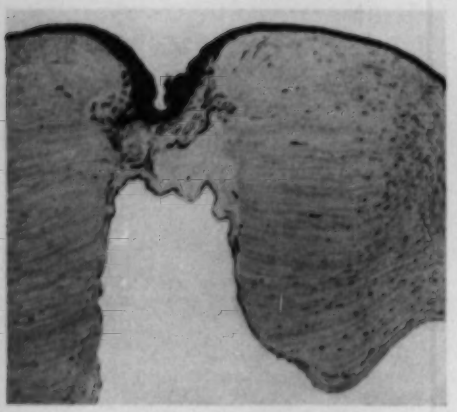

Fig. 14C (McDonald and Wilder). Histology of eye shown in Figure 14A. Note marked stromal thickening and fibroblastic inhibition.

ter one week characterized by more pronounced gaping of the wound and a translucency of the tissue covering the uveal tissue. This was a constant finding in all of the 10 rabbits studied.

Histology. Section of these eyes confirmed the fibroblastic inhibition produced by beta radiation at the limbus, and a greater tendency toward incarceration of the iris in the posterior defect (figs. 15A and B).

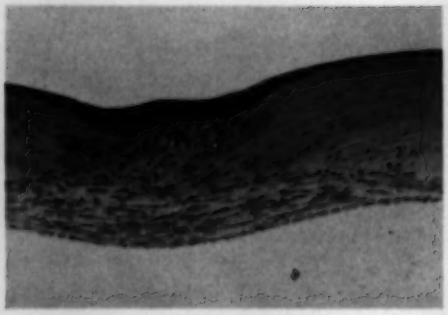

Fig. 14D (McDonald and Wilder). Histology of control eye.

Comment

In these studies there was no evidence of any acceleration of wound healing which has been reported by some to follow small doses of X rays applied to the skin. It may be that the dosage range utilized in these experiments was too high to demonstrate this reported effect.

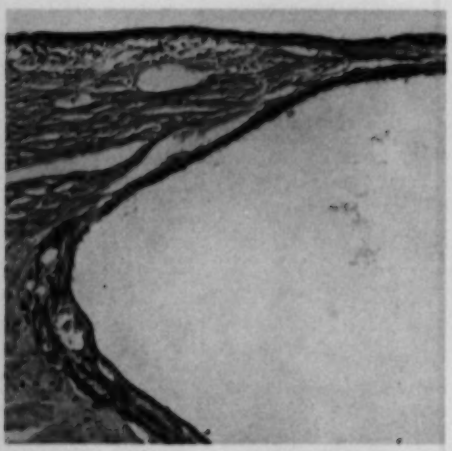

Fig. 15A (McDonald and Wilder). 5,000 rep. just prior to surgery at limbus; two weeks postoperative. Paucity of fibroblasts was a constant finding in these cases; also there was a greater frequency of iris prolapse in the radiated group.

Fig. 15B (McDonald and Wilder). Control eye. Good fibroblastic healing is seen.

These results have obvious clinical implications since the doses used in these experiments are the same or less than doses of beta radiation commonly used for elimination of corneal vascularization prior to corneal transplantation. We have some evidence for delayed healing of corneal transplants performed at the Illinois Eye and Ear Infirmary and the Research and Educational Hospitals in cases which have been irradiated preoperatively.

In one case (E. A.), the area of the graft and the recipient bed seemed to melt away during the immediate postoperative course, giving a clinical appearance very similar to that which occurred in our rabbits.

In another case (W. B.), a dehiscence of the wound developed between the graft and the recipient cornea in an area which had beta treatment preoperatively. To obviate this danger, we now avoid the use of beta radiation preoperatively, and use it postoperatively only if cortisone is ineffective in preventing vascularization of the graft, and only after several weeks postoperatively when the wound has healed securely.

Our experimental studies indicate that beta might be useful in promoting better filtration following glaucoma procedures by producing delay in healing of the limbal incision. Except for the two spontaneous filtering blebs which developed following a cataract extraction in previously radiated eyes, we have no other clinical evidence for this suggestion.

The finding was unexpected that beta radiation, given as long as three months to two years previously, still inhibited fibroblastic proliferation, in spite of no clinical evidence of corneal damage. It is unknown in what way such irradiated tissue is abnormal in its response to surgery and for how long.

Conclusions

1. Beta radiation, in doses commonly used clinically, delays healing of the corneal stroma in the rabbit following incisions through the central cornea or limbus.

2. This interference with wound healing seems to be dependent primarily on an inhibition of fibroblastic proliferation.

3. Radiation given as long as three months prior to surgery shows this effect to a similar degree as when given immediately before surgery.

4. The clinical implications of these findings are discussed.

1853 West Polk Street.

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Discussion

Dr. William F. Hughes, Jr. (Chicago, Illinois): It seems very unusual that, unlike epithelium (which recovers from the effects of radiation after several days, and in which cumulative effects do not occur, if the doses are spread out longer than two weeks), here we have, apparently, effects on the corneal stroma for as long as three months after the irradiation. Perhaps it is even longer, as we found out in clinical cases of transplantations in which grafts do not seem to heal well, even though the beta radiation had been given many months before.

I do not believe this is the entire solution for the filtering procedures which scar over. Beta radiation might be effective in preventing the scarring of the limbal incision; but, so far, it has not been demonstrated to prevent the adhesion of the conjunctiva and Tenon's capsule to the underlying sclera, in other words, closing off the bleb itself.

Dr. Derrick Vail (Chicago, Illinois): May I ask a question? I am a fish out of water in all this experimental work; but I recall some studies made by von Sallmann, in working for the NRC, on the effect of cysteine in the prevention of some damage done to the lens.

I should like to ask whether the essayists have conducted an experiment comparable to that using cysteine as a preventative of this very exciting finding?

Or, if they have not, would they consider such an addition?

Dr. James E. McDonald (Chicago, Illinois): We have used cysteine in an experimental study to see if its protective effect could be demonstrated on radiation burns of the cornea. In a group of rabbits some would be pretreated with subconjunctival cysteine and both the treated eyes and their untreated controls would be subjected to a high dose of beta radiation. Those with subconjunctival cysteine showed burns less often and of less severity than the unprotected controls. Dr. von Sallmann has done a great deal of work with cysteine. I believe that the measure of protection is such that in pretreatment with cysteine the effective dosage is reduced by about 35 or 40 percent. I do not know whether it has any practical significance as in the case of atomic attack in reducing the radiation deaths. I must emphasize that the protective effect of cysteine is only seen when the cysteine is given before radiation.

VIRUS ISOLATIONS FROM PATIENTS WITH KERATOCONJUNCTIVITIS*

Ann M. C. Fowle, Ph.D., Anne Cockeram, B.A.,
and H. L. Ormsby, M.D.
Toronto, Ontario

Sanders and Alexander (1943) were the first to report the isolation of a strain of virus of epidemic keratoconjunctivitis. They used tissue cultures of minced embryonic-mouse-brain to adapt the virus to mice. Interest in this virus was renewed when Ruchman (1951) and Cheever (1951) showed the very close relationship of Sanders' strain of virus of epidemic keratoconjunctivitis and that of St. Louis encephalitis. During the past few years Braley (Cockburn, 1954) found that Sanders' strain was no longer neutralized by sera from patients recovered from epidemic keratoconjunctivitis to the extent it had been previously. Cockburn et al. (1953) found no neutralizing antibodies against either Sanders' strain of epidemic keratoconjunctivitis virus or the virus of St. Louis encephalitis in sera from patients in two small epidemics in the mid-west. Ormsby and Fowle (1954) also failed to show any neutralization of the Sanders' strain of virus with sera from convalescent patients from an epidemic in Windsor, Ontario, in which 549 persons developed the disease.

Sezer (1953) reported the isolation of the virus of epidemic keratoconjunctivitis from

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patients in Turkey. In this isolation the virus was propagated on the chorioallantoic membranes of hens' eggs. Sezer was able to adapt the virus to the egg membrane by grafting human corneal tissue onto the chorioallantoic membrane for the first passage in eggs. Mice were subsequently infected with the ground membrane and developed symptoms similar to those described by Sanders (1943). Japanese workers (Braley, 1953) have claimed eight other isolations of the virus of epidemic keratoconjunctivitis. But the relationship of their viruses to the etiologic agent on this continent is still unknown.

Cockburn (1954), in a comprehensive review of the present status of epidemic keratoconjunctivitis, points out that "a satisfactory strain of the causal agent is not available in this country" (p. 484). In the following report three isolations of virus are described, from patients with keratoconjunctivitis, each with slightly different clinical manifestations of the disease.

Materials and Methods

Tissue cultures

Roller tubes of monkey-kidney epithelium were obtained from the Connaught Medical Research Laboratory, Toronto. The cells were maintained after inoculation in 1.0 ml. of synthetic medium No. 697 (or slight modification thereof*) containing penicillin (1,000 units per ml.) and streptomycin (500 µg. per ml.).

HeLa cells in roller tubes were supplied by Microbiological Associates, Bethesda, Maryland, and were maintained after inoculation in 1.0 ml. of maintenance medium supplied and the above antibiotics.

Human corneas (Fowle and Ormsby, 1955) taken from cadavers at the time of autopsy were implanted in shallow plasma clots in Carrel flasks and covered with medium composed of 30 percent horse serum and five-percent chick embryo extract in Earle's salt solution and containing the above antibiotics.

Roller tubes of human-kidney epithelium were supplied by Dr. A. J. Beale of the Virus Laboratory of the Research Institute, Hospital for Sick Children, Toronto.

For histologic examination, the cells were grown on the surface of a coverslip inserted in a roller tube. At appropriate intervals after inoculation of the cultures the cells were fixed in a solution composed of 94-percent methyl alcohol, five-percent formalin, and one-percent acetic acid, and then stained with Harris' hematoxylin (50 percent) and eosin.

Titrations

Titrations of virus pools were carried out in HeLa cell cultures by making serial 10-fold dilutions in gelatin-buffered saline. Four tubes containing 0.9 ml. medium were inoculated with 0.1 ml. of each dilution. The CPD_{50} was calculated using the method of Karber.

Neutralization tests

Neutralization tests were carried out using a constant dilution of virus and varying amounts of serum. In these tests the virus suspensions were diluted to 10^{-3} (100 CPD_{50}) in gelatin-buffered saline. Two-fold dilutions of serum were used. The virus-serum mixtures were incubated in a 37°C. water bath for 30 minutes, then 0.2 ml. aliquots of the mixtures were added to each of three or four HeLa cell cultures containing 0.8 ml. of medium. The end-point was taken 48 hours after the virus controls showed a complete cytopathogenic effect.

Results

First isolation

Case 1

History. Patient I. A., a man aged 23 years, gave a history of having been in a swimming-pool nine days previous to the onset of conjunctivitis in the right eye. The

* Supplied by Dr. A. Franklin, Connaught Medical Research Laboratory, Toronto.

initial symptoms were a foreign body sensation under the upper lid and marked tearing. There was moderate follicle formation in the lower fornix with a velvety pseudomembrane extending over the palpebral conjunctiva of both upper and lower lids. The periauricular gland on the right side was palbable and somewhat tender. On the fifth day after onset, at the height of the conjunctival reaction, two small punctate staining areas were seen on the right cornea near the pupillary margin. There was diffuse stromal thickening deep to Bowman's membrane in these areas and the patient was conscious of a haziness of his vision. These opacities were not plaque-like and sharply circumscribed. The second eye became involved in a similar process on the fifth day but the course in this eye was mild and no corneal changes developed. Ten days after the onset of the disease, the symptoms had largely subsided. At no time was there any pharyngitis, muscle pain, or malaise. Laboratory studies revealed a heavy lymphocytic reaction in conjunctival scrapings without growth of bacterial pathogens on blood agar.

Isolation technique

On the third day after the onset of the disease tears were collected and washings were taken in 0.5 ml. of gelatin-buffered saline from the right eye of the patient. These were inoculated immediately into roller tubes of monkey-kidney epithelium. Five days later the cells showed an extensive cytopathogenic effect (CPE) characterized by a rounding of 70 to 80 percent of the epithelial cells. The cells were scraped off the sides of the tubes into the fluid medium, then ground well in a Ten Broeck mortar, centrifuged, and the supernatant fluid used to inoculate new roller tubes of monkey-kidney epithelium. In this way a strain of the infectious agent was readily established.

The infection of monkey-kidney epithelium in roller tubes followed a typical pattern. The peripheral cells always rounded first, two or three days after infection, then gradually more and more of the cells became involved until five or six days later all the cells were rounded. Clumps of such round cells were interspersed with clear areas (fig. 1). Some of these clumps or individual cells became dislodged into the medium. Microscopically, the round cells were usually large, with a distinct cellular edge. The cytoplasm was quite granular, especially around the nucleus, and in some cases the nucleus became obscured with granules.

Preparations of monkey-kidney epithe-

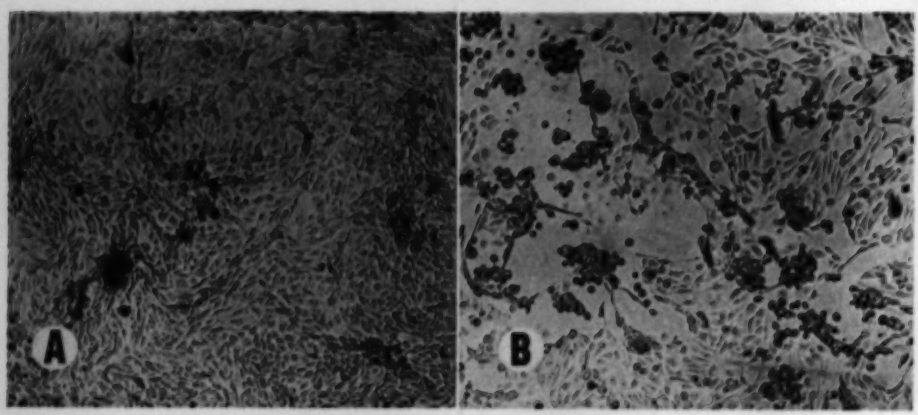

Fig. 1 (Fowle, Cockeram, and Ormsby). A tissue culture of monkey kidney epithelium (A) before and (B) four days after inoculation with the virus. Note the rounding and clumping of infected cells. (×40, unstained.)

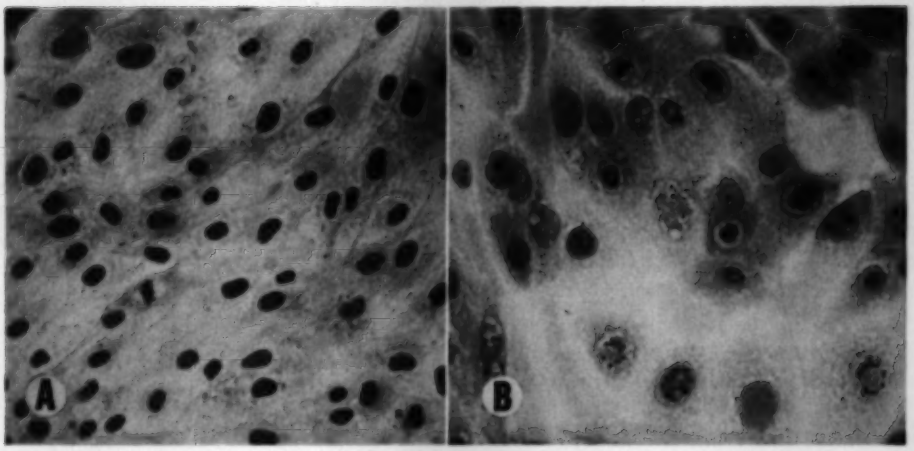

Fig. 2 (Fowle, Cockeram, and Ormsby). A tissue culture of monkey kidney epithelium (A) before and (B) four days after inoculation with the virus. Note the nuclear abnormalities. (×259, hematoxylin and eosin.)

lium stained with hematoxylin-eosin showed the following characteristics:

The infected cells stained very heavily with hematoxylin and often the nucleus became indistinguishable owing to the heavy purple staining of the cytoplasm. The nuclei of the infected cells showed a number of abnormalities (fig. 2). Most nuclei were swollen. In some there was a clear margin inside the nuclear membrane with the nuclear material forming a basophilic inclusion bodylike structure in the center. In others the nuclear chromatin showed margination or fragmentation, and the membrane was often wrinkled or no longer distinguishable. The nucleoli were present in most cells, often with a halo around them, and were eosinophilic in character. There were no inclusions in the cytoplasm.

Other susceptible tissues

A few other tissues which were tested showed a cytopathogenic effect after inoculation with the agent.

HeLa cells proved to be very susceptible, the incubation period being only 24 to 48 hours compared with five to seven days for monkey-kidney epithelium inoculated with the same concentration of the agent. With higher dilutions, the incubation period in HeLa cells was extended as long as 14 days. The cytopathogenic effect in HeLa cells consisted of granulation and rounding of cells and a clumping together of masses of these rounded cells. Later, small and large masses of clumped cells floated off into the medium. This dislodging of cells was more marked in HeLa cells than in monkey-kidney epithelial cells. Another typical characteristic was the marked lowering of the pH of the medium in the infected tubes at the time of complete cytopathogenic effect, as compared with the controls. HeLa cells proved to be the most susceptible of the tissues investigated and were therefore used for titrations and neutralization tests. The titer (CPD_{50}) in the supernatant fluid was stabilized at 10^{-6} after six passages in HeLa cells.

Human corneal epithelium was also susceptible to infection with the agent. The first indication of infection was the appearance in 24 hours of large holes in the previously intact sheet. Few or no round cells were obvious until the third day when the cells around the edges of the holes became rounded. By the fifth day most of the cells

in the sheet had rounded and formed small groups of rounded cells, and a few had become dislodged.

Human-kidney epithelium was inoculated and proved to be susceptible but at the time of writing not enough work has been done to determine the susceptibility relative to other tissues.

Tests for the exclusion of herpes-simplex virus

To rule out the possibility that the herpes-simplex virus had been recovered, cultures of rabbit corneal epithelium were inoculated. The cells showed no evidence of the cytopathogenic changes described by Doane, Rhodes, and Ormsby (1955) as characteristic of this virus. Further, the cornea of a rabbit was scarified and inoculated with the agent. No lesions were visible on the cornea upon examination with a slitlamp on the second to fifth days after inoculation, when stained with fluorescein.

Inoculation of mice

Tears and washings taken from the patient's eye on the third day of the disease were inoculated intracerebrally (0.03 ml.) into four mice weighing 12 to 14 gm. No symptoms of illness, or deaths, occurred during observation for a period of one week. Two brains were harvested and passed for five "blind" mouse passages but no illness or deaths occurred in any of the mice.

On several occasions, adult mice (12 to 14 gm.) were inoculated both intracerebrally (0.03 ml.) and intraperitoneally (0.1 ml.) with supernatant fluid from HeLa cell tissue cultures having a titer of 10^{-5}. Half the mice received a subcutaneous injection of 5.0 mg. of cortisone acetate (Merck & Company) prior to inoculation with the test material. In no instance did the mice show any symptoms of disease.

Suckling mice were also inoculated with similar tissue culture fluid intracerebrally (0.03 ml.) but did not receive cortisone. No deaths occurred and no symptoms were recognizable.

In an attempt to adapt the virus to mice, embryonic-mouse-brain tissue cultures were inoculated using a technique similar to that described by Sanders and Alexander (1943). Two series were tried. In one, the virus was alternately passed from monkey-kidney epithelium to embryonic-mouse-brain and back again for four such passages. At each passage, the embryonic-mouse-brain cultures were ground and inoculated into adult mice intracerebrally. No detectable illness was observed in the inoculated mice after four passages in tissue culture. In the other series, one set of culture flasks of chopped embryonic-mouse-brain was inoculated and maintained for three weeks. The tissue culture medium was changed twice a week and inoculated into roller tubes of monkey-kidney epithelium to test for the presence of virus. After seven days, there was no detectable virus in the medium using this technique. However, 14 days after the original inoculation, virus was again present. At this time, and four and seven days later, the tissue from duplicate flasks was ground in a small amount of medium and inoculated into mice. No symptoms of illness or deaths occurred in any of the mice inoculated.

Inoculation of eggs

The chorioallantoic membranes of 10-day-old hens' eggs were inoculated with 0.2 ml. of supernatant fluid from HeLa cell cultures having a titer of 10^{-5}. The membranes were harvested, ground, and passed to the chorioallantoic membranes of new eggs every three or four days, for four passages. No observable changes, such as pox, were visible macroscopically on the membranes. At each passage, fluid from the ground membranes was also inoculated into roller tubes of monkey-kidney epithelium to test for the presence of virus, but there was no observable cytopathogenic effect in the tissue cultures from any of the passages.

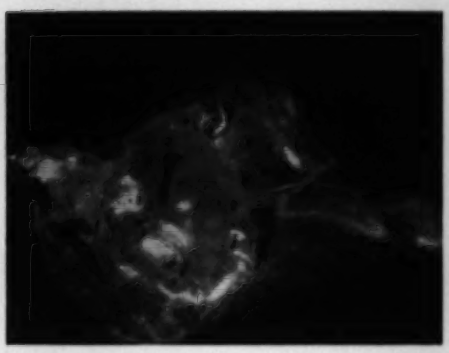

Fig. 3 (Fowle, Cockeram, and Ormsby). A chorioallantoic membrane three days after inoculation, showing a large pox, 1.0 mm. in diameter. (Egg passage, No. 2.)

When human cornea was implanted on the chorioallantoic membranes before inoculation, using the technique described by Sezer (1953), large pox (1.0 mm.) were visible on the chorioallantoic membranes at the second egg passage. Ground membrane was also inoculated into roller tubes of monkey-kidney epithelium at each passage. Even though no pox appeared on the membranes at the first egg passage, a cytopathogenic effect developed in the tissue cultures inoculated with the ground membranes. Monkey-kidney epithelial cultures inoculated with second passage material showed a complete cytopathogenic effect in three days. The serial egg passages were continued for four passages with no difficulty. Although at each passage not all the membranes had the large pox, they did exhibit a definite cloudy appearance and were very thick. Inoculation of kidney roller tubes with ground membrane from each egg passage resulted in a typical cytopathogenic effect.

Mice were inoculated with ground egg membrane from the second passage. Of 10 mice injected with 0.03 ml. intracerebrally, one showed symptoms suggestive of middle ear infection in 24 hours. By 48 hours, the mouse had a hunched back and ruffled coat and was circling almost constantly. The brain was harvested, ground, and passed to 30 mice. Of these, three showed similar symptoms within 48 hours. On the third passage, however, no mice showed any typical characteristics.

Inoculation of human volunteer

To determine whether the isolated agent would produce the characteristic symptoms of the disease, a human volunteer was inoculated with the virus from tissue culture fluid.

CASE 2

History. Tissue culture fluid from the fourth passage in monkey-kidney epithelium was inoculated into the lower fornix of the left eye of a human volunteer (H. O.), a man aged 43 years, with no previous history of keratoconjunctivitis. Five days and 12 hours after this inoculation there was a rather sudden onset of a foreign-body sensation under the upper lid of this eye and profuse tearing commenced. On the following day the preauricular node was enlarged and tender and there was a pseudomembrane on the lower conjunctiva. Scrapings revealed a heavy lymphocytic response. No inclusion bodies were seen. No pathogenic organisms were recovered on culture. Five days and 12 hours after the onset of the disease in the left eye, a similar process commenced in the right eye. At that time, slitlamp examination by a number of ophthalmologists revealed three diffuse fairly centrally placed subepithelial opacities in the left eye and one in the right eye. There was some blurring of vision in the left eye which persisted for about 10 days. Two months later, one opacity is still evident in each eye, but each is small, discrete, and off the visual axis. All symptoms of the disease had subsided by the 16th day.

Re-isolation of the virus. On the first and second days of the eye infection, tears and washings were taken and inoculated into roller tubes of monkey-kidney epithelium. Two days later, an early cytopathogenic ef-

fect was just visible in the cells at the edge of the sheet. The infection proceeded slowly, until five days later most of the cells were involved. On the third day of the eye infection, tears were inoculated into roller tubes of HeLa cells. Two days later all the cells had completely rounded in a typical cytopathogenic effect. The agent was propagated further in HeLa cells, since these cells were more susceptible than monkey-kidney epithelium. By the sixth passage it had a fixed titer of 10^{-8}, similar to that of the original isolation.

Mice were inoculated intracerebrally with tears and washings taken on the second day, but showed no visible signs or symptoms of illness during observation for a period of one week. The brains were harvested and passed for five "blind" mouse passages, but no illness or deaths occurred in any of the mice.

Neutralization tests

Neutralization tests were carried out with both viruses using two-phase sera from the patient, I. A., and the human volunteer, H. O. Blood from patient I. A. was taken on the third day (acute phase serum) and the 18th day (convalescent phase serum) of the disease, and from the volunteer, H. O., on the first (acute phase serum) and 12th day (convalescent phase serum) of the disease. Serum was also available from H. O., taken three months prior to infection.

The results of the neutralization tests are presented in Table 1. The values given are the dilution of serum protecting HeLa cells against a cytopathogenic effect. The results indicate a rise in neutralizing antibody in both patients to the isolated viruses.

TABLE 1

RESULTS OF NEUTRALIZATION TESTS

Patient	Preinfectious	Acute	Convalescent
I.A.	—	less than 1/4	1/32
H.O.	less than 1/2	less than 1/2	1/32

SECOND ISOLATION

CASE 3

History. Patient W. W., a man aged 26 years, an intern in a local hospital, gave a history of having examined a child with a sore eye some days previous to the development of a sore right eye himself. Examination three days after onset of symptoms revealed a follicular conjunctivitis with profuse tearing and a slightly enlarged, tender preauricular node on that side. Cultures were negative from both eyes and secretion smears from the right one showed a heavy lymphocytic response. No epithelial inclusion bodies were found. When the patient was re-examined on the sixth day, the left eye had also become infected. There was no staining with fluorescein at this time. Fine fibrinous pseudomembranes were present in both eyes. On the 12th day after the onset, there were about 12 early diffuse corneal opacities in the right eye and a number of these were associated with punctate epithelial erosions which stained with fluorescein. In the left cornea there were two similar lesions. Vision was not impaired in either eye. Five months later, there were three residual corneal opacities in the right eye. These were off the visual axis and vision was not impaired. There was no staining of either cornea at this time. This patient had no systemic reaction at any time, such as pharyngitis, muscle pain, or fever.

Isolation technique

Tears and washings were taken in 0.5 ml. of synthetic medium No. 697 on the fifth day of infection, frozen, and stored at −25°C. for five months. The material was then thawed and inoculated into roller tubes of HeLa cells in 0.1 ml. aliquots. Six days later a cytopathogenic effect was just visible and this progressed until all the cells were involved 10 days after inoculation. Thus far this agent has only been passed in HeLa cells and has not yet been characterized.

THIRD ISOLATION

CASE 4

History. Patient A. B., a man aged 64 years, gave a history of having had a pharyngitis about five days prior to the onset of a severe conjunctivitis. The onset of the conjunctivitis was accompanied by a foreign body sensation in the left eye. The patient was first seen on the third day after onset, and at this time the ocular symptoms were tearing, follicular conjunctivitis, and a marked enlargement and tenderness of the preauricular node on the left side. The patient had been using aureomycin ointment for three days, so that cultures were not taken. By the seventh day, the right eye was involved. Examination now showed very slight punctate staining with fluorescein around the central region of the left cornea, but there were no corneal opacities. On the 12th day, the patient was re-examined and punctate staining with fluorescein was now present in the right cornea, and a number of early opacities were seen in the left cornea. On the 18th day there were a large number of opacities (approximately 15) in both eyes which were now becoming more sharply circumscribed and were deeper in the left cornea than in the right. There were a few punctate staining areas still present in the left cornea but the right cornea had many present. It was characteristic of many of these staining areas that the opacities were seen to form directly beneath them and, with time, the opacities became more opaque and deeply placed. Vision was impaired in both eyes.

Isolation technique

Tears and washings were taken in 0.3 ml. of gelatin-buffered saline on the third day of infection and frozen and stored at −25°C. for eight days. After thawing, the tears were inoculated into roller tubes of HeLa cells in 0.1 ml. amounts. Three days later a cytopathogenic effect was obvious in the tubes and by the seventh day all the cells were affected. Thus far, this agent has only been passed in HeLa cells and has not yet been characterized in tissue culture so that it can be compared with the other isolated agents.

DISCUSSION

Most of the characteristics of the virus from the first isolation are similar to those described by Rowe, Huebner, et al. (1953) for the adenoidal-pharyngeal-conjunctival (APC) group of viruses. Therefore, the three agents were typed immunologically by Dr. Huebner and Dr. Rowe, who reported that all three strains of virus fitted the Type 3 APC group of viruses. This is in contrast to the strain recently isolated by Jawetz and Thygeson (personal communication) from a patient from the orient, which according to Dr. Huebner is of another type immunologically. Clearly, more isolations need to be made both on this continent and abroad. The isolated viruses must be critically characterized and the symptoms of the patients closely observed, before the relationship can be clarified of the keratoconjunctival virus infections and the adenoidal-pharyngeal-conjunctival ones.

The difficulty encountered in infecting mice with these agents is in direct contrast to the findings of both Sanders (1943) and Sezer (1953). This discrepancy may be due to the present mild, nonepidemic nature of the disease and, therefore, either a modified strain of the virus or some other virus may be the causative agent in this area. None of the APC group of viruses has yet been found to be infectious for mice or other laboratory animals (Rowe et al., 1953, and Huebner, personal communication, 1955).

SUMMARY

1. A virus was isolated in tissue culture by inoculation of roller tubes of monkey-kidney epithelial cells or HeLa cells with tears and eye washings from the infected eyes of three patients with keratoconjunctivitis.

2. The virus from one patient was investigated more fully than the others, with the following results:

The virus produced a distinctive cytopathogenic effect in monkey-kidney epithelial cells, HeLa cells, human-corneal epithelial cells, and human-kidney epithelial cells.

The virus was adapted to the chorioallantoic membrane of the hen's egg by grafting onto it human corneal tissue.

It was not possible to fix the virus in mice, but a transient illness did occur in mice inoculated with infected ground egg membrane.

The virus produced a mild, but characteristic, disease in a human volunteer and was re-isolated in tissue culture.

There was a rise in neutralizing antibody in the serum of both the original patient and the human volunteer, as shown by neutralization tests using HeLa cell tissue cultures.

The virus failed to produce pathologic changes on the cornea of a rabbit or in rabbit-corneal epithelium growing in tissue culture.

3. The three isolated viruses were found immunologically to fit the Type 3 adenoidal-pharyngeal-conjunctiva group of viruses.*

Banting Institute.

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* A discussion of this paper follows the paper. "Studies of immunity in vaccinia keratitis in rabbits." (see page 199.)

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TISSUE CULTURE TECHNIQUES*

In the study of herpetic infections of the eye

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Improvements in tissue culture techniques during the past few years have advanced the study of virus diseases by making available more rapid diagnostic methods. Infection of the cornea due to the viruses of herpes simplex, vaccinia, variola, and epidemic keratoconjunctivitis still present difficulties in diagnosis to the clinician, and it now seems possible, through tissue culture methods, to differentiate these diseases by the laboratory investigation of eye washings.

This study was undertaken to develop a more sensitive test for the virus of herpes simplex. Parker and Nye[1] in 1925, using hanging drop cultures of rabbit testis, were the first to grow the virus in tissue culture. At present the three tissues which appear to be most advantageous in the isolation of the herpes simplex virus are rabbit corneal epithelium,[2] the human carcinoma strain HeLa,[3] and human kidney. The present paper reports our experience with these tissue culture systems in the study of herpetic infection.

Material and Methods

Preparation and maintenance of cultures

a. *Media.* The following media were used in the preparation and maintenance of tissue cultures:

1. *Natural feeding mixture*
   Hanks' balanced salt solution .....3 parts
   Ox serum ultrafiltrate .....1 part
   Beef embryo extract 2.5%
   Horse serum 2.5-5.0%
2. *HeLa propagation medium*
   Hanks' balanced salt solution 60%
   Human serum 38%
   Chick embryo extract 2%
3. *HeLa maintenance medium*
   Hanks' balanced salt solution 87.5%
   Ox serum ultrafiltrate 10%
   Horse serum 2.5%
4. Synthetic Medium No. 199,[4] with the addition of five-percent horse serum, was used in the preparation of human kidney cultures. Horse serum was omitted in human kidney maintenance medium.

Penicillin and streptomycin were added to all media at a concentration of 250 units and 100 μg. per ml., respectively.

b. *Tissue.* Thin slices of epithelium were shaved from the corneas of freshly killed rabbits, and were chopped into fragments approximately one mm. in diameter. Three fragments were placed directly on the wall of a pyrex test tube which had been warmed at 37°C. for several hours. All tubes were then refrigerated at 4°C. for 30 minutes.[5] A quantity of 1.5 ml. of "Natural Feeding Mixture" with five-percent horse serum was added, and the tubes were rotated in a roller drum at 37°C. By three days a good growth of epithelium was usually present around the fragments. At this time the medium was replaced by one ml. of "Natural Feeding Mixture" containing two and one-half-percent horse serum.

HeLa cell cultures were prepared according to the method of Syverton, Scherer, and Elwood.[6] One-ml. quantities of HeLa cell suspension (approximately 2 by 10^4/ml.) were dispensed in roller tubes, which were then placed horizontally in a stationary rack in a 37°C. incubator. By the fifth to seventh day a monolayer sheet of epithelium had grown out along the inner surface of the tube. The nutrient medium was changed, and the tubes were rotated at 37°C. When ready for use, cultures were washed thoroughly with three changes of Hanks' balanced salt solution to remove traces of human serum,

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and Hela maintenance medium was added.

Human kidney cultures were prepared by a modification of the method of Dulbecco and Vogt[7] from surgical or fresh post-mortem material, using Medium No. 199 with five-percent horse serum. A monolayer sheet of epithelial cells formed in seven to 14 days. The medium was then changed and the tubes were rotated.

Tissue cultures to be used for histologic examination were prepared on 11 by 40 mm. coverslips which were inserted in roller tubes. Three ml. of nutrient medium were added to coverslip preparations which were placed horizontally in a stationary rack at 37°C. until ready for use.

Histologic examination of cultures

Coverslip preparations were fixed for one to 24 hours in a solution of methyl alcohol (94 cc.), formalin (5.0 cc.), and acetic acid (1.0 cc.). Staining was by a 50-percent solution of Harris' hematoxylin (10 to 20 minutes) and a 0.5-percent alcoholic solution of eosin (30 to 60 minutes). Following a rapid dehydration in alcohols to xylol, the cultures were mounted in "Malinol" mounting medium (G. Grubler & Company, Montreal, Canada).

Virus isolations

Specimens to be tested for herpes-simplex virus were taken in Hanks' balanced salt solution, and consisted of washings and scrapings from the conjunctival sac of patients with keratitis, mouth washings or swab rinsings from patients with stomatitis, and vesicle fluid from patients with herpetic-like skin lesions. Penicillin (500 units per ml) and streptomycin (200 μg. per ml.) were added, and the specimens were left for 30 minutes at room temperature. They were then inoculated into each of four rabbit cornea cultures (0.25 ml. per tube), or stored at −40°C. until required. When possible, HeLa cell and human kidney cultures were also used. Inoculated cultures were rotated in a roller drum at 37°C. and were examined microscopically at frequent intervals.

The herpes-simplex virus used was strain H51, which was isolated from a patient showing central corneal ulceration.[8] It has been maintained by intracerebral inoculation of adult mice.

Serum neutralization tests

All sera were stored at −40°C. When ready to be tested, the thawed sera were inactivated at 56°C. for 30 minutes. Serum dilutions were prepared, and 0.5 ml. of each dilution was mixed with an equal volume of H51 virus suspension at a concentration of approximately 100 CPD_{50}. After one hour at room temperature, 0.1 ml. of each serum-virus mixture was added to each of five roller-tube cultures containing 0.9 ml. of maintenance medium. Serum and virus controls were prepared in parallel.

Results

Changes following inoculation with the stock strain of herpes-simplex virus

Rabbit cornea. Preparations examined in the wet state exhibited recognizable signs of infection 12 to 14 hours after inoculation with strain H51. After this time, infection was evident by the presence of focal areas of rounded, refractile cells. Large swollen cells, frequently separated from the intact portion of the sheet, were common in the later stages of infection. These cells consisted of a clear outer area which encircled several round, granular masses. By 30 hours the entire epithelial sheet consisted of round cells, some in clusters and others separate and swollen.

Changes were detected several hours earlier in stained preparations of rabbit cornea. Coverslip cultures removed at six to eight hours showed one or two eosinophilic nuclear inclusions surrounded by an unstained halo. Some infected nuclei showed margination of the chromatin around the nuclear membrane. By the 10th hour many cells contained a nuclear inclusion with a somewhat

different appearance. These inclusions filled the entire nucleus and stained either purple or dull pink. In some instances chromatin was scattered over the surface of the inclusion. From about the 12th hour degenerative changes in the epithelial sheet appeared rapidly, with the formation of clumps of deeply eosinophilic infected cells as well as numerous characteristic multinucleate cells. As infection proceeded, the remaining cells became increasingly pyknotic.

HeLa cells. Living cultures of HeLa cells showed no changes until almost 24 hours after infection. Small round refractile cells appeared at this time, and by 60 hours the entire sheet was composed of these cells. Some swollen cells were present, but in very small numbers (fig. 1).

Stained preparations of infected HeLa cultures showed changes at about 15 hours, these being several hours later than those first seen in rabbit cornea cultures which had been inoculated in parallel. Eosinophilic, type-A inclusions were found at this time, and by 30 hours the sheet was no longer intact, but consisted of continuous clumps of cells containing inclusion-filled nuclei, both with and without halos. An occasional swollen cell could be found.

Human kidney. Cultures in the wet state showed cytopathogenic changes within 30 to 40 hours. These changes were similar to those seen in tissue cultures of rabbit cornea, the infected cells becoming rounded and occasionally swollen.

Studies on stained preparations of infected human kidney were limited. Nuclear inclusions and giant cells typical of herpetic infection were common.

Application to the diagnosis of herpetic infection

Although tissue cultures of rabbit cornea, human kidney, and HeLa cells appeared to be equally susceptible to strain H51, attempts to isolate a virus from collected specimens were carried out using rabbit cornea because of the short time elapsing before this tissue showed the typical cytopathogenic changes.

In a survey of 20 cases of suspected herpetic infection it was found that specimens from 13 of these produced characteristic changes in tissue cultures of rabbit cornea which were identical to the cytopathogenic changes described above (fig. 2). Eleven of the 20 cases were tested in parallel on the scarified cornea of a rabbit. The results are given in Table 1. It will be seen that in no instance were signs of infection evident in the rabbit eye without a corresponding change in tissue culture. Of particular importance is the fact that, in three cases, characteristic changes were seen in inoculated cultures, but not in the corresponding inoculated rabbits. Furthermore, a positive response could be detected readily in most

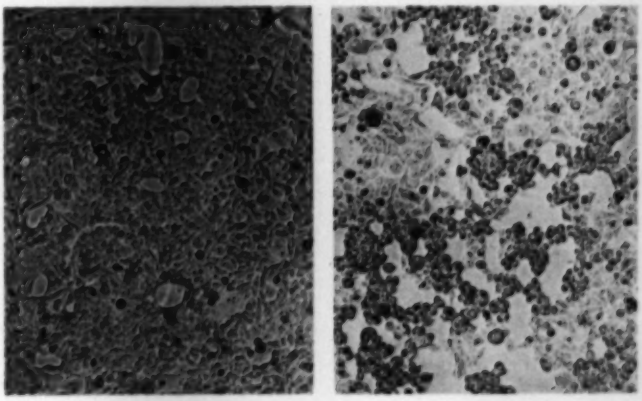

Fig. 1 (Doane, Rhodes, and Ormsby). Tissue cultures of HeLa cells at 12 days. (Left) Normal. (Right) Forty hours after infection with herpes simplex virus. Cells appear round and clumped. Note occasional swollen cell. (×40.)

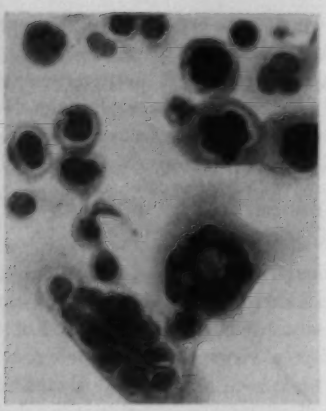

Fig. 2 (Doane, Rhodes, and Ormsby). Tissue culture of rabbit cornea 20 hours after inoculation with washings from a case of stomatitis. Note the giant cells containing clusters of nuclei with inclusions. (Hematoxylin-eosin, ×259.)

unstained tissue cultures within 12 hours, and in stained preparations within eight hours, whereas a positive reaction in the rabbit eye was not visible for at least 30 to 40 hours. A laboratory report, therefore, based on the findings in inoculated cultures was possible within about 12 hours after receipt of the specimen. A report based on the findings in inoculated rabbits was not possible for seven to 10 days, due to the long histologic procedure required for the cornea in toto.

TABLE 1

LABORATORY FINDINGS IN CASES OF SUSPECTED HERPETIC INFECTION

Clinical Picture	Case Reference Number	Presence of Changes Characteristic of Herpes	
		In Tissue Cultures of Rabbit Cornea	On Rabbit Cornea *In Vivo*
Stomatitis	2, 10, 14	+	+
	22	+	−
	12, 13	+	NT*
	7	−	−
Keratitis	19	+	+
	16, 20	+	−
	9	+	NT
	11, 15, 17	−	−
	6, 8, 26	−	NT
Skin Lesions	24, 27, 28	+	NT

* NT = not tested.

Experiments were conducted to determine whether the agent isolated in the collected specimens was, in fact, the virus of herpes simplex. Tissue culture fluids from positive cultures were put through four successive passages, resulting in the appearance of characteristic changes at each stage. Positive fluids were also passed to rabbits and suckling mice, resulting in each case in a recognized herpetic response. Several of these fluids were tested against a serum prepared by intravenous inoculation of rabbits against strain H51. All fluids tested were neutralized by this immune serum.

Application to the Study of Antibody Responses

The techniques described provide a relatively simple rapid method for the determination of herpetic antibody in human serum. Acute and convalescent phase sera from a number of patients from whom herpes-simplex virus had been isolated were tested in tissue cultures against the H51 strain. The neutralizing antibody level of these sera was demonstrated by their ability to neutralize the virus, thereby inhibiting the usual cytopathogenic changes seen in infected cultures.

Discussion

While the techniques described above enable the laboratory to investigate more fully the cytopathogenic changes which occur in herpes-infected tissues, and provide a rapid diagnostic method which is more sensitive than the standard method of rabbit cornea inoculation, there are problems which still remain to be solved in the laboratory diagnosis of herpetic infections.

It will be seen in Table 1 that specimens collected from patients with stomatitis or vesicular lesions of the skin showed herpes simplex virus in nine of 10 cases. Of the 10 specimens collected from typical cases of

dendritic keratitis, virus was recovered only from five. It appears, therefore, that the present method may need further modification if we are to uncover the latent virus, or the virus which has been made inactive by local antibody or through some undetermined process.

Summary

1. Tissue cultures of rabbit corneal epithelium, human carcinoma cells (strain HeLa), and human kidney have been used in the laboratory study of herpetic infection.

2. Within a few hours after infection with the virus of herpes simplex, tissue cultures showed characteristic nuclear inclusions and giant cells.

3. Tissue cultures of rabbit corneal epithelium were used in the laboratory diagnosis of clinically typical herpetic infections.

4. Herpes-simplex virus was isolated from nine of 10 specimens collected from patients with either stomatitis or skin lesions; whereas, virus was recovered from only five of 10 cases of keratitis.

5. It appears that tissue cultures of rabbit corneal epithelium are more sensitive than the rabbit cornea in vivo for the isolation of herpes-simplex virus.

6. Tissue culture techniques were found to be applicable to the study of neutralizing antibody response in patients with herpetic infection.

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STUDIES OF IMMUNITY IN VACCINIA KERATITIS IN RABBITS*

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Infections of the cornea due to the viruses of herpes simplex, vaccinia, variola, and epidemic keratoconjunctivitis remain a serious problem in ocular diseases. Several investigators in the past have emphasized the importance of circulating antibodies in the development of corneal immunity to virus infections.

Sabin[1] showed that vaccinia virus failed to multiply when added to normal testicular cells in tissue cultures which had been bathed previously in immune serum and subsequently washed once with saline. He also found that virus elementary bodies neutralized with immune serum regained their infectivity after centrifugation and resuspension in saline. From these observations he concluded that tissue cells were capable of picking up protective substances from immune serum which resulted in local tissue immunity.

Camus[2] reported that intracorneal injections of specific immune serum conferred corneal immunity to the vaccinia virus. Rivers, Haagen, and Muckenfuss[3] demonstrated that vaccinia failed to infect corneal epithelium in tissue cultures which had been previously incubated in immune serum. Rhodes and van Rooyen[4, 5] have stated that rabbits, hyperimmunized by intravenous injections of vaccinia, were completely protected against a subsequent corneal inoculation with the virus.

The role of local tissue antibodies in the immune response to infection has been stressed by other workers. Hartley[6] injected vaccinia virus into specially prepared skin and found that antibodies developed at the site of inoculation in a high concentration, frequently before appearing in the general circulation. Thompson[7] injected foreign protein into the cornea and later demonstrated the presence of a high concentration of antibodies in the excised cornea. These local antibodies persisted after their disappearance from the general circulation. Hall, MacKneson, and Ormsby[8] investigated this problem using herpes-simplex virus and concluded that serum antibody "played a small but important part in reducing the severity of herpetic keratitis," whereas "local defense mechanisms play the major role."

Vaccinia is a very suitable virus for immunologic studies because of its ability to stimulate antibody formation. It has been used in this investigation to evaluate the degree of corneal immunity produced by various immunizing procedures.

MATERIALS AND METHODS

Rabbits were used as experimental animals and the course of corneal infection was followed by observation with a hand slit-lamp. The virus used for corneal vaccination was manufactured by The Connaught Laboratories for immunization against smallpox. The virus is sealed in equal quantities in fine glass tubes, thus ensuring a constant infective dose. Before vaccinia virus was placed on the cornea, local anesthesia was obtained with pontocaine and the epithelium was scratched with a needle. Virus used for intravenous injection was rabbit lapine, prepared from the crusts of three day-old vaccinia lesions. Serologic immunity was measured by the complement fixation reaction using the method outlined by Craigie and Wishart.[9]

In order to assess the effect of various immunizing procedures, the primary take following corneal vaccination has been divided into four stages (fig. 1).

Stage I. Following scarification the defects in the corneal epithelium healed completely within 24 hours. After 48 hours numerous

* From the Departments of Ophthalmology and Pacteriology, University of Toronto.

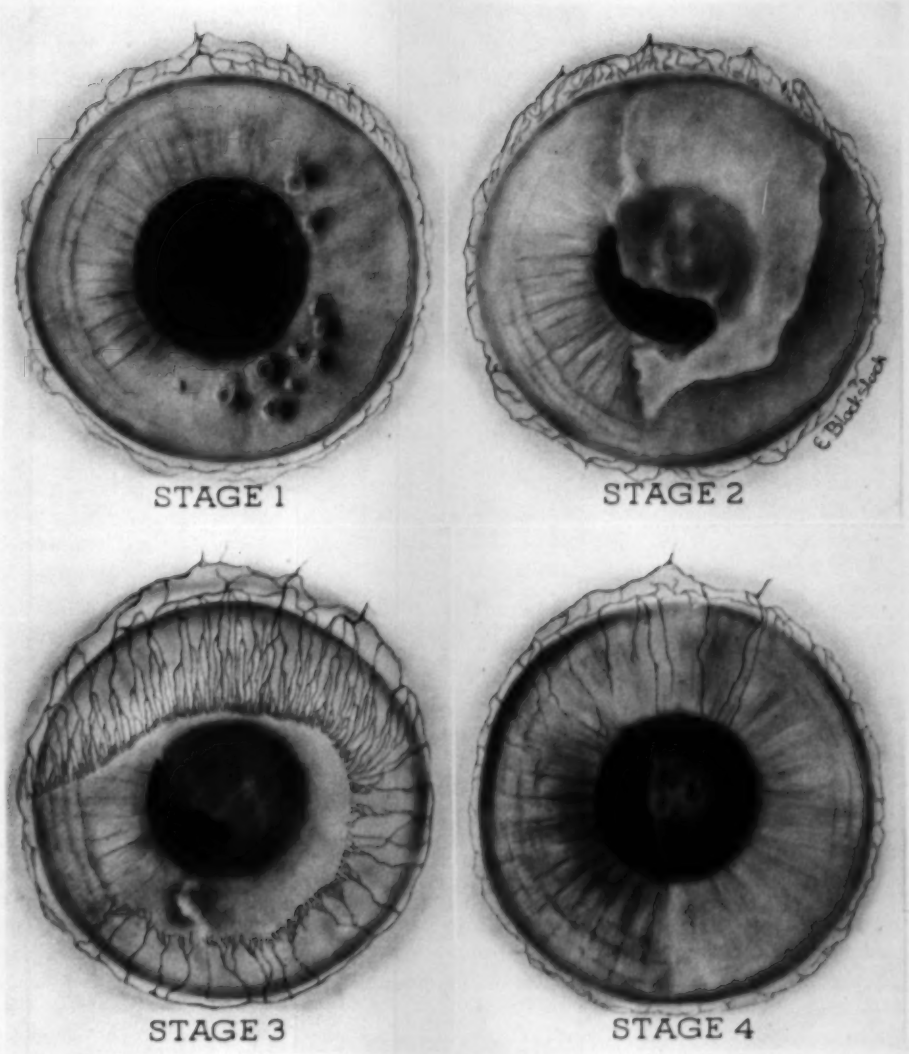

Fig. 1 (Speakman and Ormsby). Primary corneal infection with vaccinia virus.

tiny, discrete, punctate epithelial erosions appeared, which could only be seen in the early stages after the instillation of fluorescein.

Stage II. The multiple corneal ulcers remained discrete for three or four days and then became confluent, so that at the height of stage II frequently over half the corneal epithelium was denuded. As the ulcers progressed the corneal stroma lost its translucency and iris details were obliterated. The conjunctival vessels became congested, and there was marked swelling of the lids and profuse sticky secretion.

Stage III. Corneal vascularization occurred at about the 10th day. As the new vessels progressed toward the center of the cornea, the general reaction of the eye subsided, the corneal ulcers healed, and stromal edema cleared from the periphery toward the center.

Stage IV. The healed cornea was characterized by varying degrees of vascularization and scarring. As this stage progressed, the vessels tended to become obliterated. At the end of several months many eyes were free of open corneal vessels, and only a few areas of speckled stromal scarring remained.

Procedure

Experiment I

Effect of corneal vaccination on corneal immunity

Fifteen rabbits were vaccinated in the right eye. At the end of one month the first group of five of these were revaccinated in both eyes, the second group at two months, and the third group at three months.

Group I: Revaccination after one month

The right eyes of the first group of rabbits showed little evidence of infection following revaccination. In most cases there was a slight loss of corneal translucency which lasted for about 48 hours. This was associated with a transient dilatation of the corneal vessels. In two of the five rabbits a mild stage I reaction developed but this did not progress. Thus there was a very high degree of protection upon reinoculation of a recently infected eye.

Infection in all left eyes of this first group progressed from stage I to stage III. In each case there was considerable secretion and edema of the eyelids. In spite of this severe reaction, the intensity of the primary reaction was slightly modified since the extent and duration of the ulceration were reduced. When both eyes were examined three months after inoculation the amount of residual scarring and vascularization in the two eyes was very similar for each rabbit.

Group II: Revaccination after two months

All the right eyes of the second group of rabbits developed multiple corneal erosions and confluent ulcers. The persisting corneal vessels dilated rapidly. However, the ulcers healed more rapidly and were not as extensive as those seen following primary vaccination. These results suggested that after two months the residual immunity in the previously inoculated cornea was low.

The left eyes of the second group of rabbits all developed severe infections which could not be differentiated from a primary take. The residual scarring and vascularization in both eyes of each rabbit were comparable.

Group III: Revaccination after three months

The results in this group resembled those noted in the group reinfected at two months.

Serology studies

In the initial phases of this experiment blood samples were taken at monthly intervals following vaccination. Subsequent testing showed that this interval was too great for accurately recording the fluctuations in antibody levels. Consequently a second group of three animals was vaccinated in the right

eye, and blood was removed more frequently for complement fixation determinations. Complement fixing antibodies first appeared in the serum about 10 days after infection and reached a peak in 15 to 20 days, with titers varying from 1:4 to 1:32. Ten days after reaching a maximum level the titers rapidly decreased to zero, subsequently rising to lower levels than those at the peak. Revaccination of both eyes when the titers were maximal did not result in higher antibody levels. However, revaccination one month after the peak was followed by a rapid rise in titer which lasted for 15 days (fig. 2).

At 20 days, when complement fixing antibodies were maximal, revaccination of both eyes produced a modified primary take in the left eyes, but in the right eyes a stage I reaction was seen in only one of three animals, indicating a high degree of immunity.

Revaccination of both eyes of these same animals at 57 days, when the serum complement fixing antibodies were low, produced a transient dilatation of the corneal vessels and slight corneal edema. Since local corneal immunity at this particular stage did not parallel the serum antibody levels, it was necessary to consider the possibility that some other factors were modifying the reaction.

Experiment II

Effect of corneal vascularization on corneal immunity

Since vascularization invariably followed corneal vaccination, an attempt was made to assess the importance of this factor in the development of corneal immunity. Four rabbits were vaccinated on the skin and at the same time 0.1 cc. of horse serum was injected into the cornea of each right eye. Two weeks later the dermal vaccination and the horse serum injections were repeated. A severe allergic keratitis developed which resulted in extensive vascularization of the right eyes.

When the hypersensitivity reaction of the cornea had subsided at the end of a month, and the vessels had begun to atrophy, both eyes were inoculated with vaccinia. In the vascularized right eyes there was a rapid dilatation of the corneal vessels, but in spite of this a stage I reaction developed. Later small confluent ulcers were seen which did not involve large areas of the cornea and which consequently healed rapidly. The diffuse corneal edema also cleared quickly.

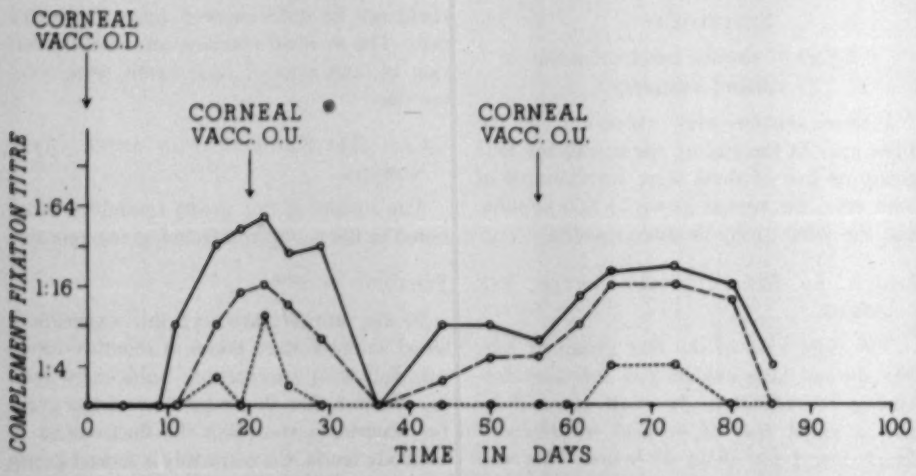

Fig. 2 (Speakman and Ormsby). Complement fixation titers following corneal vaccination.

In the left eyes a much more severe inflammation developed. The primary ulcers of stage I were more numerous and spread more rapidly. The stage II reaction was much more severe than in the right eye, and stage III was still developing while reaction was clearing in the right eyes. One month after corneal vaccination when both eyes were compared, there was less scarring and vascularization in the right eyes. Thus vascularization of the corneas of the right eyes resulted in a reduction in the severity of the reaction, but did not protect the cornea from infection.

Experiment III

Effect of hyperimmunization on corneal immunity

It was seen in experiment I that vaccination of the normal cornea at the height of the immune response from a previous infection resulted in a slight modification in the reaction. An attempt was therefore made to stimulate additional circulating antibody to see if complete protection of the cornea could be obtained. Ten rabbits were skin vaccinated, and following the initial serologic response were given repeated intravenous injections of living virus. It will be seen from Figure 3, which illustrates the response of two representative animals, that complement fixing antibodies increased rapidly after intravenous virus injections.

When the titers of two animals had reached 1:64 and 1:128 the right eyes were inoculated, and since both developed severe keratitis additional intravenous injections were given. The final titers obtained were all close to 1:256, and in one animal the titer reached 1:512.

In spite of these high levels of serum antibody, stage I reactions were seen in all eyes 48 hours after corneal vaccination. By the fourth day the primary ulcers had become confluent, but they were much shallower than those seen in primary infections, and the edges were irregular. Stromal edema in this stage was also reduced. At the end of five days the ulcers were beginning to decrease in size, and at the end of eight days several had healed completely. New corneal vessels developed in all these eyes but did not invade the corneas extensively. Hyperimmunization, therefore, did not result in complete corneal immunity but produced a

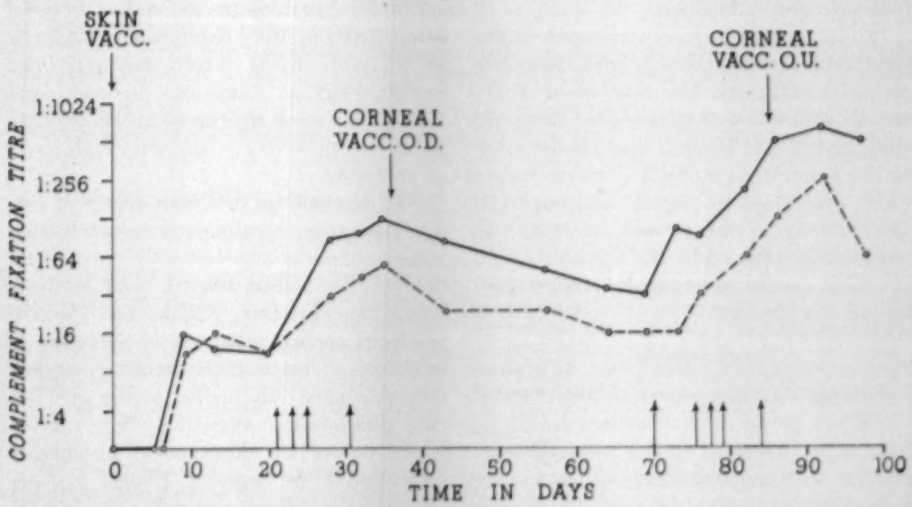

Fig. 3 (Speakman and Ormsby). Complement fixation titers following intravenous virus injection.

considerable modification of the primary infection.

Experiment IV

Effect of subconjunctival injections of vaccinia virus on corneal immunity

Six rabbits were vaccinated on the skin and after an interval of eight and 11 days subconjunctival injections of Connaught vaccinia virus were made in the right eye of each animal. Precautions were taken to avoid injury to the cornea. After each injection the conjunctival sac was irrigated with water to remove excess virus, and the eyes were carefully examined with the slit-lamp for evidence of infection. Three days after the last injection the corneas were vaccinated. Blood was tested for complement fixing antibodies at regular intervals.

The injections of virus produced mild congestion and edema of the conjunctiva which subsided in three or four days. One rabbit developed a corneal ulcer after the first injection, which was followed by vascularization and scarring. In the remaining five animals the corneas remained clear. The complement fixation titers varied from 1:4 to 1:32, the range one would expect to find following skin vaccination.

Primary corneal ulcers developed in the right eyes of only three animals following corneal vaccination. The number of ulcers varied from two to four and they remained quite superficial. The left eyes on the other hand all developed multiple punctate lesions which progressed to stage II and stage III. The severity of the corneal infection was considerably reduced by the subconjunctival injections and in two animals complete protection was obtained.

Discussion

Although immune serum protects corneal epithelium grown in tissue culture from vaccinia virus (Rivers et al.[3]), serum antibodies even in high concentrations did not confer this protection in vivo. Our inability to confirm the work of Rhodes and van Rooyen,[4,5] who reported complete corneal protection following hyperimmunization, may be due to differences in the technique of corneal inoculation. A marked reduction in the number of stage I ulcers was seen in our experiments, in a group of hyperimmune animals which were vaccinated without using local anesthetic. Furthermore, when only a few ulcers developed, they frequently healed completely in four or five days. Rhodes and van Rooyen[4,5] used light general anesthesia and examined the eyes of three animals histologically on the sixth, seventh, and 10th days. It is possible, if only a few ulcers developed, that healing occurred before the animals were killed, and infection was consequently overlooked.

The high degree of corneal immunity resulting from a recent vaccinia keratitis is only partly explained by new vessel formation. The work of Hartley[6] and Thompson[7] suggests that local tissue antibody formation was an important factor in these experimental results. In support of this view was the finding of significantly increased levels of complement fixing antibodies in extracts of corneas removed after a recent vaccinia infection. Local tissue immunity persisted after serum antibody had fallen to low levels at the end of one month, but after two months local immunity also declined. Constant exposure of the cornea to the infective agent was necessary to maintain high levels of immunity.

The development of a high degree of corneal immunity following subconjunctival injections of virus may be another manifestation of the stimulation of local antibody formation. However, Nagano and Furano[9] produced corneal immunity by intracorneal injections of inactivated vaccinia virus, and they attributed this finding to the interference phenomenon, since the effect was obtained before the development of immune bodies would be expected. Henle[10] has emphasized the difficulty in differentiating the

interference phenomenon from a local immune response when immunologically related viruses are employed. Therefore it seems unwise to draw definite conclusions regarding the exact mechanism by which immunity is acquired after subconjunctival injections of virus.

If corneal immunity in man is similar to that of the rabbit, dermal vaccination alone is not likely to result in complete protection of the cornea against variola and vaccinia infections. Subconjunctival injections of virus may be of practical importance in immunizing corneas which are subject to recurrent virus infections.

Conclusions

1. Serum antibody to vaccinia virus in the rabbit reached moderate levels after either skin or corneal inoculation, and these levels were sufficient to confer a slight degree of resistance to a subsequent inoculation of the intact cornea.

2. Hyperimmunization of rabbits by intravenous injection of live vaccinia virus resulted in high serum antibody titers which produced a further modification of the corneal infection, but did not give complete protection against the virus.

3. The highest degree of corneal immunity was seen in eyes which had recently recovered from a vaccinial keratitis and in eyes which had received subconjunctival injections of virus.

4. Vascularization of the cornea following keratitis due to horse serum injections increased the resistance of the eye to the virus, but this was not as great as that following a previous corneal vaccination.

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Discussion

Dr. Alson E. Braley (Iowa City, Iowa): I think these two papers* are too good to let go without making some comment. I think perhaps I will limit my remarks to asking a few questions of Miss Doane, if I may.

First, she has demonstrated very well a method of isolation of herpes virus which is far better than our methods. But herpes is a virus, as she has shown, which is quite easily isolated, even by the cruder methods we have always used; these new, finer techniques which are now available are much better, and show the virus very readily in the tissue cultures.

I believe these particles represent colonies of the virus which she has quite well demonstrated in the nuclei of the cells.

The failure of corneal inoculation, however, has

* See also "Tissue-culture techniques in the study of herpetic infections of the eye" (Frances W. Doane, A. J. Rhodes, and H. L. Ormsby), page 189.

always been very interesting to me. Recently, in attempting to do some other antibody studies, using rabbit cornea with herpes virus, we have found that a group of rabbits fed on antibiotic-containing rabbit food were almost nonsusceptible to inoculation with herpes virus. I wonder if she knew, or if she checked to see, if her rabbits had been fed on antibiotic food.

The second paper, on vaccinia, represents a study in immunology which I have also been very much interested in and, I believe, represents very well the rapid fall of complement-fixing antibodies. The complement-fixing antibodies and the neutralizing antibodies of herpes and of vaccinia are most interesting.

While I believe that, at the present time at least —I may change it next week—the circulating or neutralizing antibodies represent or may have some part to play in the recurrent infections which can occur, the hyperimmune animal certainly had very high not only complement-fixing antibodies, but must have had high neutralizing antibodies. These animals took the infection as well on their corneas.

As I told Dr. Speakman yesterday, Jenner knew that the cornea was not protected from vaccinia infection after skin inoculation; that the cornea would still take the vaccinia infection.

The reason for this, of course, he did not know; and I am not certain of it even yet. Certainly, the part played by local immunity is most important; also, the local immunity, I think, may play a very important part in herpes as well as in vaccinia, so that these studies done by Dr. Speakman are certainly important so far as immunity in vaccinia and in herpes are concerned.

Just one more point; that is that the failure of isolation of the virus from the cornea in cases of dendritic keratitis only represents the balance which is so often present between the local immunity and the amount of virus which is available for culture inoculation. What you are doing in many of these instances is collecting as much antibody (let us call it) of one type or another, local antibody, which immediately neutralizes the virus and therefore will not infect a tissue culture. That is one of the main reasons for failure in herpes infection, particularly when they begin to go into what I call the metaherpetic phase, or the chronic type of herpetic infection.

It has been a real pleasure to listen to all of these papers; and I am simply delighted that someone else is finally becoming interested in the most important phase of ocular infections, that of the virus diseases.

DR. FRANCES W. DOANE (in closing): As far as I know, Dr. Braley, all the rabbits have been fed normal rabbit-pellet diets, with no antibodies in their food.

DR. BRAILEY: It is not easy to buy animal food now which does not contain antibiotics. You have to get them especially from a company which makes animal food that does not contain an antibiotic.

DR. DOANE: I have to admit I do not know.

STUDIES ON THE ETIOLOGY OF EPIDEMIC KERATOCONJUNCTIVITIS*

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San Francisco, California

Epidemic keratoconjunctivitis is an eye infection which has occurred in large outbreaks in Europe, the Far East, Hawaii, and on the Pacific Coast. The epidemics of 1941-42 in the San Francisco area centered around shipyards in war time,[1] but during the past decade the disease has recurred both sporadically and epidemically (outbreaks often originating in physicians' offices.[2])

The most constant clinical features appear to be the following: After an incubation period of seven to 10 days there develops an intense follicular conjunctivitis with marked conjunctival injection. Often a thin pseudomembrane is formed. The conjunctival involvement, occasionally unilateral, is accompanied by enlarged and tender preauricular lymph nodes. There is a scanty nonpurulent exudate, with abundant lacrimation. Five to 10 days after the onset of conjunctivitis, corneal lesions develop. These are characteristically small, round, subepithelial corneal opacities, generally without significant superficial ulceration, and without impairment of corneal sensitivity. The number and size of these corneal opacities vary, as does their interference with visual acuity. These opacities disappear

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slowly in from a few weeks to two years.

The etiology of epidemic keratoconjunctivitis has not been established. Bacteriologic studies have been negative uniformly.[1] The viral agent described by Sanders in 1942[2] may have been lost or mislabeled. His preserved "keratoconjunctivitis virus" is closely related to, or identical with, St. Louis encephalitis virus[4, 5] and appears to bear no relation to the present disease. Maumenee et al.[6] isolated another agent from a typical case which proved to be a herpes simplex virus. Preserved acute and convalescent sera from patients of the 1941 epidemic showed no rise in antibody titer against either of these viruses.[7] Other claims for the isolation of etiologic agents of epidemic keratoconjunctivitis were reviewed by Cockburn[7] but none has been confirmed.

In view of the recent resurgence of epidemic keratoconjunctivitis in several parts of the world[8] it was decided to restudy the entire problem of etiology in keratoconjunctivitis. The present report summarizes the initial results in our attempts to isolate viruses from the eye and in antibody studies in cases of epidemic keratoconjunctivitis and herpetic keratitis.

Materials and Methods

Specimens

Scrapings of conjunctiva and/or cornea were taken from the area of maximal involvement in patients with active conjunctivitis or keratitis. A sterile platinum spatula was used and the material suspended by vigorous agitation in HeLa maintenance medium (10-percent chick serum in medium 199), bathing HeLa cell tissue cultures.* Previously these HeLa cultures had been washed twice with Hanks' balanced salt solution.† Part of each specimen was frozen and kept at −20°C. in HeLa maintenance medium until inoculated later into HeLa cell cultures and onto chorioallantoic membranes of 11- to 12-day-old embryonated eggs. HeLa cell cultures were incubated at 30°C. for 22 days, inspected daily by 20- to 100-fold magnification for cytopathogenic changes, and the maintenance medium was changed at intervals of four to six days. Chorioallantoic membranes (CAM) were harvested 48 to 72 hours after inoculation, inspected for pocks, and if questionable lesions were observed the material was passed at least once. Herpes viruses isolated in tissue culture or on chorioallantoic membranes were submitted to neutralization tests with specific antiserum prepared in rabbits.

Serologic methods

One group of sera, obtained in a 1951 epidemic of epidemic keratoconjunctivitis in Windsor, Canada, was kindly made available by Dr. A. Fowle and Dr. H. L. Ormsby. A second group of acute and convalescent sera collected in 1954 in Chicago was generously provided by Dr. M. D. Pearlman. A third group of convalescent sera collected two years after a 1953 outbreak was provided by Dr. Irving Leopold of Philadelphia. Sera from patients observed in this clinic were handled by a standard manner[9] and stored frozen at −20°C. until used.

Neutralization tests

Neutralization tests with herpes viruses *in ovo* were carried out by a method described earlier.[9] For neutralization tests in tissue culture equal parts of inactivated serum dilution and virus dilution were mixed, incubated for 45 minutes at room temperature, and 0.2 ml. of the mixture inoculated into each of two twice-washed HeLa cultures to which 0.8 ml. of maintenance medium was then added. The tubes were incubated in stationary position at 36°C. without change of medium and inspected daily for cytopathogenic effects. Readings were taken for at least four days after complete degeneration of the control tubes (virus + normal rabbit serum) had

* Obtained from Tuskegee Institute, Alabama.

† All tissue culture media were obtained from Microbiological Associates, Washington, D.C.

taken place. Tubes were read blindly and cytopathogenic changes were graded from 0 to ++++. Agreement between tubes of the same group was good. The result was considered definite neutralization only when there was a difference of +++ in readings for more than two consecutive days.

Neutralization tests with "Trim" virus met with considerable difficulties identical with those described by Rowe et al.[19] Different batches of HeLa cells infected with the same virus pools showed marked variations in incubation periods and degree of cytopathogenic effects. In certain tests even sera of known neutralizing capacity had very little effect; conversely, normal rabbit serum delayed cytopathogenic effect for one or even two days. In view of the low titer of "Trim" virus, only undiluted or twofold to 10fold diluted tissue culture fluid could be used as viral inoculum. Small variations in technique did not improve the results significantly. Consequently, it was necessary to include positive and negative standard control sera and virus titrations in each test. Many runs of neutralization tests had to be discarded, and only those tests in which all controls were satisfactory are reported.

Complement fixation tests

An antigen of RI 67 virus (APC type 4) was generously made available by Col. T. Berge in the form of tissue culture fluid. This was stored at −20°C. until used. All sera were inactivated at 60°C. for 30 minutes. Two units of antigen (tissue culture fluid dilution of 1:8) and two units of complement were employed with two full units of amboceptor and saline as diluent, with 18-hour fixation at 4°C. The above RI 67 antigen was not anticomplementary. An antigen from pooled fluids of HeLa cell cultures inoculated with "Trim" virus was used in a dilution of 1:4. This antigen was frequently anticomplementary and had to be heated at 56°C. for 30 minutes for satisfactory tests.

Results

Virus isolations

To date, attempts have been made to isolate viruses from cornea and conjunctiva in 66 individuals. Of these, 10 were "normals," that is, persons who showed no evidence of external eye disease, and 11 others had some form of conjunctivitis or keratitis, but did not present a clinical picture suggesting either herpetic or epidemic keratoconjunctivitis. No cytopathogenic agent was isolated in HeLa cultures from any of these 21 persons, nor were transmissible lesions produced on chorioallantoic membranes. Thirty-nine patients were given a clinical diagnosis of "herpes-simplex keratitis," and from them eight strains of typical herpes-simplex virus were isolated. Strains of herpes simplex were also isolated from two of the patients with keratoconjunctivitis as described below.

Five patients with definite and one with questionable epidemic keratoconjunctivitis were studied. Two of these had had the acute disease in 1953, and no virus isolations had been attempted then or during the present study. The antibody titer of their sera obtained 16 to 17 months after the acute illness was 1:10 and 1:40 respectively. The remaining four patients in this group were observed to have active clinical eye disease.

Patient "Trim," a merchant seaman travelling between the Orient and San Francisco, was the first individual with acute epidemic keratoconjunctivitis seen during the present study. He had an intense conjunctivitis with pseudomembrane formation (fig. 1) and enlarged preauricular nodes. The right eye was involved first and the left four days later. His subepithelial corneal infiltrates progressed to form the round lesions typical of epidemic keratoconjunctivitis (fig. 2). His illness developed while he was on board ship and he remembered no definite exposure. He was seen in our clinic on the 12th day after onset. Scrapings taken

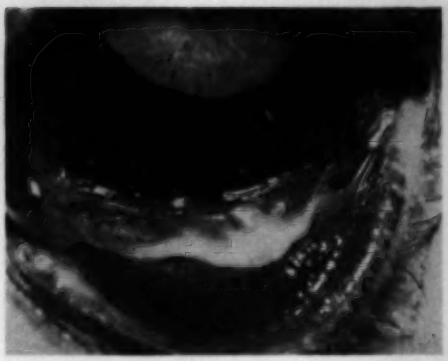

Fig. 1 (Jawetz, et al.). "Trim," L.E. Pseudomembrane on lower tarsal conjunctiva—12th day of acute keratoconjunctivitis.

Fig. 3 (Jawetz, et al.). "Cott," R.E. Clinical photograph of subepithelial corneal infiltrates six days after onset.

from both conjunctivas yielded a cytopathogenic agent in HeLa cultures on the 21st day of incubation, described below as "Trim" virus.

The patient "Trim" was hospitalized and attended by the nurse "Cott." Ten days after her first contact with patient "Trim," "Cott" developed an acute conjunctivitis of the right eye, with markedly enlarged, tender preauricular nodes. The left eye remained entirely normal, but the right eye gradually developed the unequivocal subepithelial round corneal infiltrates of epidemic keratoconjunctivitis (fig. 3). Scrapings obtained from patient "Cott" on the sixth day of her illness yielded no virus in HeLa cultures, but from scrapings obtained on the 14th day a typical herpes simplex virus was recovered. The patient "Cott" showed a marked antibody titer rise to virus "Trim" during the period of her illness, as indicated below.

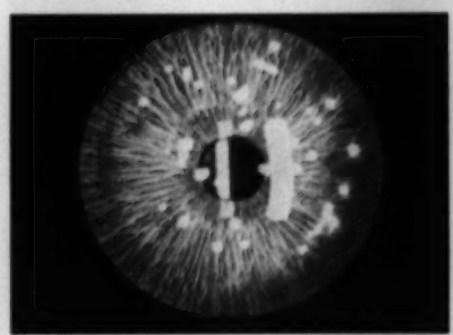

Fig. 2 (Jawetz, et al.). "Trim," L.E. Subepithelial corneal infiltrates (artist's drawing) 99 days after onset.

A second seaman had developed conjunctivitis of one eye, which later spread to the other side about one month before his visit to the clinic. Scrapings from both eyes failed to yield any transmissible agent, although suggestive degenerative changes occurred in HeLa cultures. The patient refused to permit withdrawal of an early serum specimen. Later specimens contained antibodies to "Trim" virus. The illness of this individual is tentatively diagnosed as epidemic keratoconjunctivitis, because of highly suggestive clinical findings.

Patient "Rena," a third merchant seaman, was seen in the clinic one week after onset of an intense conjunctivitis of the right eye, and a markedly enlarged, tender, right preauricular node. Scrapings yielded a typical herpes simplex virus in tissue culture and on chorioallantoic membranes. The left eye became involved three days later. The conjunctival exudate was predominantly mononuclear in character, but the patient failed to develop the typical corneal lesions of epidemic keratoconjunctivitis. In the course of his illness he developed antibodies to "Trim"

Fig. 4a (Jawetz, et al.). Normal HeLa cell culture. Magnification, ×120.

virus, as described below. His clinical diagnosis remains uncertain.

Characteristics of "Trim"

In the HeLa cell culture inoculated with original specimen from patient "Trim," the first cytopathogenic changes occurred on the 17th day of incubation, and degeneration was virtually complete on the 22nd day. Subsequent passages in HeLa cells degenerated in two to four days when undiluted tissue culture fluid was used as inoculum. With dilution, the incubation period was greatly increased so that with inocula of 0.1 ml. of 10^{-1} HeLa tissue culture fluid the onset of cytopathogenic changes was delayed four to eight days. The TC50 never exceeded 10^{-2} dilution up to the present 12th passage. Neither early nor late passage had any influence on the titer. Disintegration of cells by repeated freezing and thawing of partly or completely degenerated HeLa cell cultures failed to increase the infection by titer, and perhaps slightly reduced it.

The cytopathogenic effects in HeLa cells were similar to those described for RI or APC viruses.[11,12] Initially the cells at the periphery of the cell sheet rounded up and fused into highly refractive clumps. Within the clumps the cells were granular and their outlines were often lost (fig. 4a, b). The clumps tended to peel off the glass, and often large segments of the cell sheet became separated and floated in the medium. The pH of HeLa cell cultures (in 90-percent mixture 199) usually remained near neutral, even upon prolonged incubation without change of medium. The acid reaction described for APC viruses[12] in HeLa cultures was not observed.

"Trim" virus was readily filterable through Seitz pads, without loss of titer, and was resistant to treatment with 30-percent diethyl ether for 18 hours at 4°C. It was not pathogenic by any route, including intra-

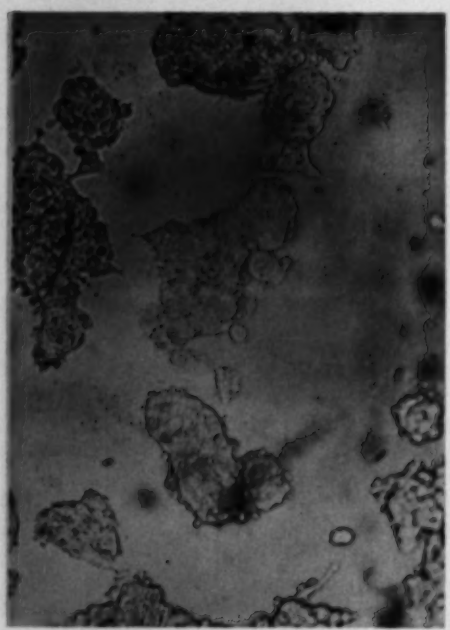

Fig. 4b (Jawetz, et al.). HeLa cell culture five days after inoculation with "Trim" virus, 10^{-4} dilution, showing typical late cytopathogenic effects. Magnification ×120.

cerebral and corneal, for suckling or adult mice, guinea pigs, or rabbits. On the chorioallantoic membrane of 11- to 12-day-old embryonated eggs questionable pocklike lesions were seen upon inoculation of infected, but not of uninfected tissue culture fluids. These appeared to be passed on chorioallantoic membranes, but re-inoculation of such membranes into HeLa cultures failed to give cytopathogenic effects. Thus, proof is lacking that the observed lesions on chorioallantoic membranes represent viral effects.

"Trim" virus had no cytopathogenic effect on tissue cultures of the following cell lines: L (mouse fibroblasts), pf 14 (rat fibroblasts) (kindly made available by Dr. G. Gey), chick fibroblasts, and HeLa cells adapted to growth in horse serum (kindly made available by Dr. R. Caillaux). A human cell line derived from an adenocarcinoma of the lung (kindly made available by Dr. A. W. Frisch) was as susceptible as HeLa cells grown in human serum.

Pools of fluid from HeLa cell cultures infected with "Trim" virus were a satisfactory antigen for complement fixation tests, after heating at 56°C. for 30 minutes. This antigen fixed complement in the presence of sera containing antibodies to the APC group of viruses,[12] thus indicating that it contained the soluble group antigen. "Trim" virus was neutralized by pooled human gamma globulin, by a homologous antiserum prepared in rabbits, and by sera from patients with epidemic keratoconjunctivitis as described below. It was not neutralized by rabbit antiserum to herpes simplex, or to St. Louis encephalitis virus (kindly made available by Dr. E. L. Lennette), and by sera from patients with herpetic keratitis. Dr. R. J. Huebner (National Institutes of Health) and Lt. Col. T. Berge (Sixth Army Area Medical Laboratory) kindly attempted to "type" the "Trim" virus. Using monotypic specific antisera against various APC types they were unable to obtain neutralization of "Trim" virus by any antiserum of types 1, 2, 3, 4, 5, 6, or 7.

The C-F group antigen clearly places the "Trim" virus into the group of APC- ARD-viruses and the virus has been classified as type 8 by Dr. Huebner.

Specific rabbit antiserum to "Trim" virus fails to neutralize APC type 3 recovered from cases of conjunctivitis.

Serologic investigations

Neutralization tests performed with 10 paired sera from patients with epidemic keratoconjunctivitis, including those from whom herpes-simplex viruses were recovered, revealed no rise in titer of neutralizing antibodies to herpes simplex. Thus it may be said that none of these patients could have had a primary infection with herpes virus coincidental with their epidemic keratoconjunctivitis.

The survey of antibodies to the newly isolated "Trim" virus yielded the results summarized in Table 1. Neutralizing antibodies in a titer of 1:10 or more were found in four patients tested from the 1951 Windsor, Canada, epidemic; in one patient tested from Canada, 1954-55; in five patients seen in California with the typical disease between 1953 and 1955; in nine patients from the outbreak in Chicago during 1954; and six patients from the outbreak in Philadelphia in 1953. Thus 25 individuals with typical epidemic keratoconjunctivitis in the period 1951-1955 had neutralizing antibodies to the "Trim" virus in a serum dilution of 1:10. Nineteen of these 25 patients had such neutralizing antibodies in serum dilutions of 1:20 or greater. In contrast, in a group of 19 patients with herpetic keratoconjunctivitis (with isolation of herpes simplex virus from seven of them) neutralizing antibodies to "Trim" virus were found in only a single case; neutralizing antibodies to "Trim" virus could be detected in only one of 10 patients with uveitis.

This rather striking apparent relationship between "Trim" virus and epidemic keratoconjunctivitis occurring in four distinct geographic areas (Canada, Philadelphia, Pa-

TABLE 1

ANTIBODIES TO "TRIM" VIRUS AMONG PATIENTS WITH KERATOCONJUNCTIVITIS OR UVEITIS

	Location	Year	No. Patients	Neutralizing Antibodies*	Definite Evidence of "Trim" Infection Pos./Total
A. Typical epidemic keratoconjunctivitis	Canada	1951	4	4	
	Canada	1954–55	1	1	
	California	1953–55	5	5	25/25
	Chicago	1954	9	9	
	Philadelphia	1953	6	6	
B. Atypical keratoconjunctivitis	Canada	1955	4	0	1/5
	Asia-Calif.	1954	1	1	
C. Herpetic keratoconjunctivitis	California	1949–55	19	1	1/19
D. Uveitis	California	1954–55	10	1	1/10

* Number of patients whose serum neutralized 50–100 TC 50 of "Trim" virus in a dilution of 1:5 or more.

cific-California, Chicago) is emphasized by those cases from which acute and convalescent serum specimens could be examined. As shown in Table 2, unequivocal rises in the titer of neutralizing antibodies occurred in the majority of instances. However, the peak titer in no case exceeded 1:160, whereas in other systemic infections with APC viruses[11] higher titers are common.

In infections with APC-like viruses complement fixation tests are of only limited value because all agents apparently share a soluble group antigen. Thus infection with any type of APC-like virus can result in complement-fixing antibodies. On the other hand it appears probable that complement-fixing antibodies persist for only a limited period after infection. With these reservations it is of interest that among 19 patients with typical epidemic keratoconjunctivitis all had complement-fixing antibodies for both "Trim" virus and RI 67 virus. Such antibodies were found in only five of 19 patients with herpetic keratitis and seven of 31 patients with uveitis. A majority of the patients with epidemic keratoconjunctivitis also showed a definite rise in complement-fixing antibodies during their illness.

The 1954-55 sera kindly made available by Dr. Ormsby and Dr. Fowle, Toronto, must be discussed separately. The pertinent data are summarized in Table 2 (bottom). The first of these cases, "McCul," had both a typical disease and a striking rise in neutralizing antibodies to "Trim" virus. Patients "Avot" and "Orms" had atypical forms of keratoconjunctivitis which was definitely associated with type 3 APC virus infection, and they lack neutralizing antibodies to "Trim" virus. They, as well as patient "Matt" (who developed no opacities), would have to be excluded from consideration in the present series because their illness cannot be exactly defined within the limitations previously described. The fifth patient, "Whel," is said to have "typical epidemic keratoconjunctivitis with opacities" yet has no demonstrable neutralizing antibodies to "Trim" virus.

DISCUSSION

The relationship of the laboratory findings reported in this paper to the etiology of epidemic keratoconjunctivitis is not clear. A number of possibilities must be considered:

a. Accidental viral contamination of tissue cultures. This can be safely excluded

TABLE 2

SEROLOGIC EVIDENCE OF "TRIM" VIRUS INJECTION IN PATIENTS WITH ACUTE EPIDEMIC KERATOCONJUNCTIVITIS

Geographic Location	Patient	Days after Onset	Neutralizing Antibodies[a]	Complement Fixing Antibodies[b]	Remarks
Asia-Calif.	Trim	12	?	?	"Trim" virus isolated on 12th day
		99	40	20	
		141	160	10	
California	Cott	6	0	0	Contact of "Trim" Herpes simplex virus isolated on 14th day
		80	80	20	
Chicago	Lars	3	0	20	Typical epidemic keratoconj. with opacities
		19	40	40	
Chicago	Hans	2	0	0	Typical epidemic keratoconj. with opacities
		24	80	80	
Chicago	McDow	5	0	?	Typical epidemic keratoconj. with opacities
		33	20	160	
Chicago	O'Don	8	5	0	Typical epidemic keratoconj. with opacities
		24	160	160	
Chicago	Quar	4	0	0	Typical epidemic keratoconj. with opacities
		33	80	20	
Chicago	Kotyl	3	0	80	Typical epidemic keratoconj. with opacities
		31	10	160	
Chicago	Fitt	34	80	?	Typical epidemic keratoconj. with opacities
		64	80	10	
Toronto	McCul	3	0	0	Typical epidemic keratoconj. with opacities
		74	80	80	
Asia-Calif.	Rena	7	5	20	Atypical epidemic keratoconj. without opacities. Herpes simplex virus isolated on 7th day
		92	40	80	
Canada	Avot	3	0	160	Atypical keratoconj. APC type 3 virus isolated by Dr. Ormsby
		42	0	160	
Canada	Orms	1	0	10	Atypical keratoconj. APC type 3 virus isolated by Dr. Ormsby
		27	0	10	
Canada	Matt	3	0	0	Keratoconjunctivitis without opacities
		136	0	0	
Canada	Whel	13	0	160	Keratoconjunctivitis with opacities
		180	0	160	

[a] Reciprocal of the serum dilution giving significant neutralization with 50–100 TC 50 of "Trim" virus. 0 = No neutralization in a serum dilution of 1:5.

[b] Reciprocal of the serum dilution giving +++ complement fixation with "Trim" antigen. 0 = No complement fixation in serum dilution of 1:10.

because no APC-like viruses were present in this laboratory at the time of recovery of the "Trim" agent, and in hundreds of control tissue culture tubes no herpes viruses made their appearance. Thus it is safe to assume that the various viruses originated from the patients' specimens.

b. The viruses might have been present entirely by accident in the eyes from which they were recovered, not in any way related to the acute illness. This seems plausible for the herpes-simplex viruses which

might have been latent in the patients' cornea, brought into activity by an inflammatory process of unrelated etiology. It also is a possibility for "Trim" virus, since the habitat of APC-like agents may be the conjunctiva as well as the upper respiratory tract. Yet the rise in both complement-fixing and neutralizing antibody titer to APC-like agents during the acute illness suggests that these agents at least produced an infection, albeit perhaps an asymptomatic one.

c. "Trim" virus might be the etiologic agent of epidemic keratoconjunctivitis. A stronger case for this postulate could be made if the same agent had been recovered from several clinical cases. At present the support for this claim must rest on: (1) The isolation from a typical case; (2) the almost uniform presence of neutralizing antibodies to "Trim" virus in typical cases of epidemic keratoconjunctivitis (25 out of 25) and their rarity in herpetic keratitis (one out of 19) and uveitis (one out of 10); (3) unequivocal rises in antibody titer in seven of nine patients seen during the acute illness in 1954-55.

Extensive experience with both neutral disease and experimental infection with type 3 APC virus (Dr. R. H. Huebner, personal communication) makes it very unlikely that this type of agent alone could produce typical epidemic keratoconjunctivitis. However, until the nature of the Canadian cases seen by Dr. Ormsby has been thoroughly clarified it may be necessary to withhold judgment as to the possible relationship of various etiologic agents to the morpholgic picture in eye disease.

d. Might herpes viruses alone be the cause of typical epidemic keratoconjunctivitis, as proposed by Maumenee?[6] For a number of reasons this seems most unlikely. Primary herpetic infection (including keratitis) generally occurs in childhood, and most individuals subsequently possess significant antibody levels. An epidemic spread of herpes virus from adult to adult seems exceedingly unlikely. Furthermore, herpetic keratoconjunctivitis tends to recur, whereas recurrences of epidemic keratoconjunctivitis have never been reported. The isolation of herpes virus from clinically typical epidemic keratoconjunctivitis[6] may represent a situation as encountered with patient "Cott," in the present study, but is no proof of an etiologic relationship.

e. APC-like agents, like "Trim" virus, may be an important factor in causing epidemic keratoconjunctivitis, and may be regularly present but may require another agency, like herpes virus or trauma, to produce the full-blown clinical picture. This would account for the occurrence of very mild cases in patients with only conjunctivitis in the epidemic chain[1,7] who acquire "Trim" virus infection but lack the additional agency. In the present work, the only support for this hypothesis is the isolation of typical herpes simplex virus from patients "Cott" and "Rena" while these individuals were undergoing infection with an APC-like virus, proved by serologic reactions. Might the coexistence of these two agents result in different forms, but always severe keratoconjunctivitis?

In view of the known ability of at least type 3 APC virus to produce conjunctivitis[10,12] but not keratitis, it might be postulated that, in susceptible populations, viruses like "Trim" might spread epidemically in eyes. By themselves such APC-like viruses might produce only a conjunctivitis, but acting together with other influences (for example, trauma or other viruses such as activated latent herpes simplex) they might produce the full clinical picture of keratoconjunctivitis. There is no support available at present for this hypothesis but it seems worthwhile to pursue it in future studies. The evidence presented in this paper warrants the conclusion that "Trim" virus is somehow related to epidemic keratoconjunctivitis. The nature of the relationship remains to be established.

Summary

1. A new virus was isolated from a clinically typical case of epidemic keratoconjunc-

tivitis. This virus serologically belongs to the group of APC-viruses and has been classified as type 8.

2. Neutralizing antibodies in significant concentration were found in the serum of 25 of 25 patients who suffered from typical epidemic keratoconjunctivitis between 1951 and 1955 in four distinct geographic areas in North America. Similar antibodies were found in only one of 19 patients with herpetic keratoconjunctivitis and in one of 10 patients with uveitis.

3. Typical herpes-simplex virus was isolated from a patient during the acute phase of epidemic keratoconjunctivitis, while she was undergoing infection with the APC-like agent, proved by rise in antibody titer.

4. The possible role of the newly recovered virus, and other agents, in the etiology of epidemic keratoconjunctivitis is discussed.

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Discussion

Dr. Alson E. Braley (Iowa City, Iowa): The problem of epidemic keratoconjunctivitis and the related viruses is most interesting, and a problem in which I have been very much interested for a number of years.

Likewise, there is the problem of what has been called Greeley disease. This and the epidemics reported by Dr. Ryan and his co-workers before the A.M.A. Section on Ophthalmology. Most interesting narrations have occurred with the passage of time.

In 1943, a virus was isolated by Dr. Sanders and Mrs. Alexander. At the same time, I was working with the disease. We followed much the same program which Dr. Sanders did, but we were unable to isolate the virus from our cases. However, many of our mice did become ill.

We found it extremely difficult to isolate the virus by the methods used by Dr. Sanders and Mrs. Alexander. However, our mice, when they did recover from mild infections, were not susceptible to the virus isolated by Sanders. This gave us a lead, that at least we were dealing with a similar agent.

The work of Dr. Kimura and Dr. Fowle leads us, then, to some real findings and some really important contributions to this entire problem. They have shown that they can isolate the virus on HeLa cells and in monkey kidney quite readily; but that this virus is not infectious to mice, when inoculated in mice. It also does not propagate in our tissue cultures.

One more point I would like to make is that, in studies similar to those of Sezer (that is, the egg inoculations using corneal epithelium as the medium on which the egg will grow, then inoculating that material into mice), the results show some illness in mice.

I believe this represents mutations in the virus.

At the present time, we have had the virus we call EK since 1943. We have carried it in our laboratories all these years. Admittedly, it was kept in the

deep freeze for a little over three years, while we were in service, then taken out again and reinoculated into mice. This virus no longer is neutralized by the serum from patients who have had the typical clinical picture of epidemic keratoconjunctivitis.

Our virus has been completely adapted to mice, and is no longer related at all to the present group of APC viruses, as they are described.

All of these APC viruses probably represent various types of mutations, from type 1 clear through to type 9; and it so happens that we now have a method by which easy isolation can be done.

This, then, I am sure, will show us that, while all of these viruses are related, they will bring us back, I think, to the many arguments which some of you may have heard on polio at the recent meeting here in Atlantic City, when Salk and the rest of them all presented some material. I do not know whether any of you were there; but I do not believe that we need the arguments they had there, because I think we have some of the better mutations, and we can discuss these problems together without getting into any really serious difficulties.

Our virus has been changed, and changed so completely that it is no longer even remotely associated with epidemic keratoconjunctivitis. However, it would be interesting to grow it on HeLa cells and see how it could be typed, if it could be typed as an APC group. It may actually now be a type 10 virus.

I would like to show you that many of these, I think, are related, in one way or another, to mutations which occur in influenza; and I would like to present to you two cases, those of two doctors on whom we tried to isolate a virus.

These two doctors were ear-nose-and-throat men who did not wear glasses while doing a bronchoscopy. Actually, of course, the patient coughed while they were looking, and they got something in their eyes, which they carefully washed out; but, five days and approximately 12 hours later, they came down with this lesion (slide). Here we will show the upper lids, a marked follicular conjunctivitis with a follicular pharyngopathy and, following this, a severe pharyngitis. The pharyngitis developed almost two weeks after the eye infection, during which they also had bronchitis, with high fever up to 104°F. The eye apparently was the point of inoculation for these two men.

(Slide) The next two pictures are of the other patient, who was not standing quite so close and did not get quite so much inoculum as the first one.

(Slide) A follicular conjunctivitis which lasted for a period of approximately two weeks.

I believe now that these cases probably all represent various types of changes which will produce similar clinical pictures. I do not believe, since we have never been able to get any cross-neutralization with herpes, that they are related to the herpes virus, but more closely related to the virus of influenza, which is known to produce several different types of mutations.

DR. J. H. ALLEN (New Orleans, Louisiana): I wish to congratulate the authors of both papers for beautiful presentations and I especially wish to call attention to the bravery of the human volunteer, Dr. Ormsby, who, as you know, is fully aware of the dangers involved.

Although the theory of virus mutation, as just presented by Dr. Braley, is interesting, I believe the data presented in these papers and previous ones indicate that the condition we know as epidemic keratoconjunctivitis is a syndrome which may be caused by a number of different viruses. At present it would seem that several types of the APC viruses are capable of producing almost identical clinical manifestations. Whether unrelated viruses also may produce clinically indistinguishable manifestations remains to be proven.

In this vein of thought it would have been interesting and important to have known the results of inoculation of a human volunteer with the virus isolated by Dr. Kimura's group.

DR. H. L. ORMSBY (Toronto, Canada): I do not think that this problem is quite as confused as it is being made out.

In 1951, we had an epidemic of what we called epidemic keratoconjunctivitis in the Ford Motor Plant in Windsor, Ontario. There were 600 cases of viral conjunctivitis and only 89 of these patients developed keratitis and corneal opacities. Of six convalescent sera from this epidemic, four were recently shown by Jawetz to have neutralizing antibody to the Trimborn virus. One of the interesting features of this Windsor epidemic was that, in the early months of the epidemic, there were few corneal opacities. When the epidemic reached its height there were quite a number of opacities. Finally, when the epidemic waned, there were very few opacity cases to be seen.

(Slide) This Kodachrome is of one of the patients with this type of disease which we saw in Toronto in the fall of 1951, the same year of the Windsor epidemic. Of nine patients with this type of conjunctivitis in Toronto, only one developed corneal opacities.

(Slide) This is the close-up on the same patient. You can see the very slight pseudomembranous appearance in the lower fornix.

(Slide) This is another patient who in 1951 had the subconjunctival haemorrhages which are fairly common in epidemic keratoconjunctivitis but who did not develop opacities.

In the fall of 1954 Dr. Clement McCulloch sent us this patient from his office. There was pseudomembranous formation on both upper lids and there was a subsequent development of corneal opacities typical of epidemic keratoconjunctivitis. It is rather interesting that nine days later Dr. McCulloch developed epidemic keratoconjunctivitis. The opacities in both patients are still present six months afterward. Dr. McCulloch's serum was shown by Jawetz to have a rising antibody titre to the Trimborn virus.

(Slide) This is a Kodachrome of a patient who volunteered for an experimental eye inoculation with an APC3 virus which we had isolated. Although corneal opacities developed (and persisted in one case up to six months), there was blurring of vision for only a few days. In this particular case the

opacities disappeared within six weeks. Pseudomembranes, which you will see in the next Kodachrome, were present in some cases but were easily removed and were very fine in texture.

(Slide) This is another type of viral conjunctivitis which we have seen recently which is characteristic of Beal's conjunctivitis. We have no isolation on this case and we do not know the cause.

Our APC3 disease differs in several respects from that of the 1954 Washington epidemic, since none of our patients have had any fever, only one has had pharyngitis, and transmission did not occur readily in home contacts. Since all the patients from whom we isolated virus were adults, this may account for the difference in the clinical picture.

This is a new disease in Toronto, something which we had not previously seen.

DR. ALSON E. BRALEY (Iowa City, Iowa): I have one more thing I want to say. Dr. Kimura said that there were no reports in the literature of patients being reinfected with EK virus.

He did not read the literature carefully enough, because I reported one [laughter], a Dr. Wooster, whom some of you around here may know. If his son is here, he would know.

In 1943, he had a typical EK virus which produced some corneal opacities and disappeared. I also reported that the antibodies to the virus which he had at that time were present. They disappeared. In 1948, he developed a typical epidemic keratoconjunctivitis.

DR. JAMES P. LEAKE (Washington, D.C.): I wonder if Dr. Ormsby would not consider, however, that the lack of spread is due to the lack of pharyngeal components, as in Matthew 22:14: "Many are called, but few are chosen."

DR. H. L. ORMSBY (in closing): There is a great deal that can be said on this subject, if time permitted.

Mitsui, in a recent issue of THE AMERICAN JOURNAL OF OPHTHALMOLOGY, described corneal changes in keratoconjunctivitis. He described the clinical appearance in very young children whose eyes he inoculated from typical adult cases of epidemic keratoconjunctivitis. These children developed pseudomembranes but no keratitis. Alternatively, he took eye scrapings from children who had the pharyngitis, conjunctivitis, and systemic manifestations, and inoculated adult volunteers. These inoculations resulted in the typical keratoconjunctivitis of epidemic keratoconjunctivitis. It is possible, therefore, that we may have different clinical manifestations in adults and in children.

Whether spread of this disease in children occurs by droplet infection, and in the adult by direct contact, is something we have yet to work out.

THE PENETRATION OF CORTISONE AND HYDROCORTISONE INTO THE OCULAR STRUCTURES*

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Since the initial use of cortisone in eye disease[1] its use has made numerous changes in ocular therapeutics. The effectiveness of cortisone and hydrocortisone applied locally has been particularly advantageous.[2] It has been shown that the efficacy of these steroids is due to local inhibition of the inflammatory process at its site. Therefore, it should follow that local concentration of each drug should be the principal factor in patient response. The net therapeutic result is modified by the anti-inflammatory activity of the drug actually present locally.

Dosage forms and concentrations have been until now largely based on impressions of clinical effectiveness. The desirability of a more objective method of deciding dosage form, dose, and route of administration is evident.

One possible approach to such a method would be experimental determination of local steroid concentration at the intended anatomic site of action. The attempts to do this have been based until now on the chemical determination of ketones giving the Porter-Silber reaction with phenylhydrazine.[3-9] The disadvantages of such methods include nonspecificity, insensitivity, and failure to

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distinguish between endogenous and exogenous corticoid.

In an attempt to eliminate these disadvantages, our experiments using C-14 labeled corticoids and separation by paper chromatography have been undertaken. With local application of ring labeled C-14 steroid all radioactivity must represent exogenous steroid only. When pure carrier steroids are used with an adequate chromatographic technique, the radioactivity in each fraction must be steroid and not degradation products or nonsteroid ketones. With high enough specific activity there is no problem of sensitivity of the method. In some experiments described below, sensitivity is roughly 100 times that of the best chemical methods and there is no theoretical limit to potential sensitivity.

This first series of experiments is a study of the depth of penetration, and the amount of each chemical form when cortisone acetate or hydrocortisone free alcohol are applied topically for an arbitrary time interval.

Methods

Ring labeled C-14 cortisone and hydrocortisone* were diluted with specially purified inactive material† to a concentration of 2.5 percent and 0.25 percent in the case of cortisone acetate and 2.5 percent in the case of hydrocortisone. Active and inactive material was homogenized in an Elvejhem-Potter homogenizer and held in suspension by a 1.0-percent solution of gum arabic in 1.35-percent sodium chloride.

One ml. of such a suspension was applied to the anterior corneal surface of a nembutal anesthetized rabbit with a special stirring applicator (fig. 1) for an arbitrary period of 20 minutes. After the experimental period, the suspension was withdrawn from the applicator, the cornea was copiously washed with saline solution, and the eyes were enucleated.

Fig. 1 (Hamashigi and Potts). Air-bubble stirrer vacuum applicator for steroid suspensions.

Cornea, aqueous, lens, anterior uvea, vitreous, retina, choroid, and sclera were all worked up for radioactive steroid content. This was accomplished by homogenizing in an Elvejhem-Potter homogenizer with five 5.0-ml. portions of chloroform and 50 μg. each of cortisone, cortisone acetate, and hydrocortisone. The chloroform solutions were evaporated to dryness in a stream of air at room temperature and the residue was taken up in 30 ml. of 70-percent ethanol. The ethanol solution was washed with five 5.0-ml. portions of hexane (Skellysolve B), and was then evaporated to dryness once more. The residue was taken up in a small volume of methanol and transferred to paper for purposes of chromatography.

This chromatographic procedure was done according to a modification of the method of Bush[10] in which development is carried out by the use of benzene, methanol, water (2:1:1). Our modification involves pretreatment of the Whatman No. 3 paper with a mixture of equal parts of anhydrous and water saturated ether and allowing the ether to evaporate just prior to depositing the steroid at the starting point on the paper. We allow one to two hours for equilibration after placing the paper in the chamber. Chromatography is complete in 4.5 to 5.5 hours at room temperature.

When development was complete, the paper was examined under ultraviolet illumination and the spots represented by carrier cortisone, cortisone acetate, and hydrocortisone were outlined; these areas were

* Furnished through the kindness of the National Institute of Arthritis and Metabolic Diseases, Dr. Sam R. Hall.

† Furnished through the kindness of Merck & Company, Inc., Rahway, New Jersey.

then cut out, the paper extracted five times with methanol, and the methanol residues evaporated in dishes for radioactivity counting. Counting was performed in an automatic sample changer under a thin window Geiger counter tube. Because of the relatively low activities present, long counts repeated at least three times were the rule.

The feasibility of the chromatographic separation and the ultraviolet identification of cortisone, cortisone acetate, and hydrocortisone were established by numerous experiments with pure steroids, mixtures of known steroids, and tissue extracts to which known steroids were added. Examination with ultraviolet light was controlled by using the color reaction of the 2-ketol group with alkaline silver nitrate. The fluorescent spots coincided with the silver stained spots in every case.

Results

The result of experiments with topically applied 2.5-percent C-14 labeled cortisone acetate suspensions are summarized in Table 1. The 2.5-percent suspension contained 5.3 c/m/μg and the minimum detectable amount is 0.95 μg of steroid. The 0.25-percent suspension contained 1,000 c/m/μg and the minimum detectable amount was 0.005 μg of steroid.

Two experiments were done with 2.6-percent C-14 labeled hydrocortisone free alcohol applied topically for 20 minutes. This hydrocortisone suspension contained 20.2 c/m/μg and the minimum detectable amount was 0.25 μg of steroid. Under these conditions radioactivity counts were at the limit of sensitivity of the method and gave equivocal results. Hydrocortisone with higher specific activity will be needed for such an experiment.

Discussion

That cortisone reaches the intraocular structures is amply proved by the clinical effectiveness of the drug and the work of Dr. Leopold's group. The concentrations in the various tissues and the chemical form of the steroid are not on such firm ground. The earlier estimates of 40 to 50 μg of steroid in the normal aqueous,[5,6] have re-

TABLE 1

THE INTRAOCULAR PENETRATION OF CORTISONE ACETATE AFTER APPLICATION TO THE ANTERIOR CORNEAL SURFACE

Time = 20 min.

Applied Suspension Concentration	Limit of Detectability mμg	Steroid	Tissue Content Millimicrograms						
			Cornea	Aqueous	Ciliary Body and Iris	Lens	Vitreous	Retina	Cloroid and Sclera
2.5% CA 5.3 c/m/μg	950	CA C H	3×10^3 6×10^3 —	— — —	— — —	— — —	— — —	— — —	— — —
0.25% CA 1,000 c/m/μg	5	CA C H	70 29 14(ca)	90 625(?) 30	↑ 36 ↓	— — —	— — —	— — —	↑ 187 ↓
0.25% CA 1,000 c/m/μg	5	CA C H	— — —	28 6 12	10 5 —	— — —	— — —	— — —	↑ 372 ↓
0.25% CA 1,000 c/m/μg	5	CA C H	99 69 26	40 5 6	6 12 —	— — —	— — —	— — —	13 66 8

CA = Cortisone Acetate
C = Cortisone
H = Hydrocortisone

cently been revised to < 0.5 μg,[9] and after local application of 2.5-percent suspension every 15 minutes, four times, the aqueous concentration was measured as 5.0 to 8.0 μg of cortisone acetate/ml. or roughly 1.0 to 2.0 μg per eye.[9] This compares with < 0.95 μg per eye in our experiment with 2.5-percent suspension and a mean of 40 millimicrograms with 0.25-percent suspension. There seems legitimate room for speculation here on whether the level of cortisone acetate is really in the microgram range or an order of magnitude lower. This is particularly true in view of the more intensive application of steroid and the immediate enucleation in our procedure which would be expected to give a higher, not lower, figure for the aqueous level than in the experiments of Weimar and Leopold.

Moreover, it is of great interest to note that in both aqueous and cornea there are measurable amounts of cortisone free alcohol and of hydrocortisone after application of cortisone acetate to the eye. In the cornea the quantity of cortisone free alcohol (table 2) is approximately half that of the acetate; in the aqueous the amount is approximately one sixth. Even more striking, the amount of hydrocortisone in the aqueous exceeds that of free cortisone and averages one third the amount of cortisone acetate. In the cornea it is closer to one-fourth of the acetate present.

When the poor penetration of hydrocortisone free alcohol is borne in mind this seems additional confirmation for the greater ease of corneal penetration of lipophilic substances over hydrophilic ones as described by Cogan and Hirsch[11] and by Swan and White.[12] However, one may well wonder on the basis of the rapid degradation of cortisone acetate in the eye whether the actual therapeutic agent is not hydrocortisone free alcohol present in the strikingly low quantity of 10 millimicrograms per eye.

Further, one should note that no detectable steroid was found in lens, vitreous, or retina, but that the choroid and sclera fraction did show activity. If one can rule out external contamination here, and such contamination is not likely, one can only attribute the result to the multiple anastomoses of the venous outflow of the eye. Experiments are in progress to clear up this point.

It is reasonable to bring up the question whether any significant amount of hydrocortisone acetate is present in the eye and whether this substance penetrates more easily than the free hydrocortisone. There was no hydrocortisone acetate available either labeled or unlabeled at the time these experiments were begun. We shall acetylate our hydrocortisone and look for its acetate in the eye in our next series of experiments.

TABLE 2

RATIOS OF CORTISONE ACETATE, CORTISONE, HYDROCORTISONE IN AQUEOUS AND CORNEA AFTER APPLICATION OF CORTISONE ACETATE TO THE ANTERIOR CORNEAL SURFACE

Ratio of	Tissue	Experiment No.		
		5	6	7
Cortisone / Cortisone acetate	Cornea	0.41		0.69
	Aqueous		0.22	0.13
Hydrocortisone / Cortisone acetate	Cornea	0.21		0.26
	Aqueous	0.33	0.44	0.16

CONCLUSION

1. When cortisone acetate labeled with C-14 is applied to the anterior corneal surface, radioactive steroid is detectable in cornea, aqueous, and uvea.

2. The steroids so detected can be shown to consist of cortisone free alcohol and hydrocortisone even though pure cortisone acetate suspension has been used.

3. The concentrations of all three steroids are markedly less than those previously reported by chemical methods.

4. When labeled hydrocortisone is used for such experiments, the levels of activity resulting are too low to give unequivocal results.

5. This leaves the nature of the actual therapeutic agent in local use of cortisone acetate subject to additional speculation.

Western Reserve University (6).

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Discussion

Dr. Irving Leopold (Philadelphia, Pennsylvania): This paper by Dr. Hamashige and Dr. Potts offers an accurate method for determining the penetration of steroids.

It is unfortunate that they did not have available in sufficient concentration for accurate comparison the free-alcohol form of cortisone and the acetate variety of hydrocortisone to compare to the acetate variety of cortisone and the free-alcohol variety of hydrocortisone.

Up until the present time, as they pointed out, the methods available have been chemical ones such as the phenylhydrazine reaction and the use of blue tetrazolium. Both of these methods have definite drawbacks. However, Dr. Potts and Dr. Hamashige have confirmed the earlier chemical studies in that there is penetration of these compounds, as shown by the group working with Dr. Green at Wills Hospital.

Certain difficulties with the blue-tetrazolium technique have been ironed out by the Wills group. However, some definite faults are still evident. One cannot separate naturally occurring steroids from those which have been applied to the eye with the chemical methods. I fthere are variations in the amount of normally occurring steroids during the time of the experiment one will not be able to determine the extent of this in doing the analysis by this technique. The Potts-Hamashige technique should eliminate this fault.

The chemical methods can be employed to measure differences of penetration.

The Wills group has been able to demonstrate a definite difference in penetration of locally applied steroid compounds. Variations in rate and degree of penetration among cortisone, hydrocortisone, and 9-alpha-fluorohydrocortisone have been found with these techniques. The acetate and free-alcohol forms also show differences in penetration. For example, the free-alcohol form of hydrocortisone locally applied showed greater and more rapid penetration than the acetate variety in the presence of an abraded cornea.

Clinically, this may explain the experiences of Gordon and his co-workers and Hogan, et al., who feel that the free-alcohol variety of hydrocortisone has a greater anti-inflammatory reaction for intraocular-disease processes.

Earlier, it was shown that cortisone acetate did not do as well as hydrocortisone acetate for external inflammations but, with intraocular inflammations, the cortisone acetate did about as well as hydrocortisone acetate locally applied. This might be explained by the less rapid penetration of hydrocortisone acetate as compared to cortisone acetate. It may be that the use of the hydrocor-tisone free-alcohol in local preparations would result in a better penetration of this compound, and superior clinical results.

I anxiously await the studies that this group will do with the other C^{14}-labeled compounds and the steroids, as they become available.

Dr. Albert M. Potts (in closing): I want to thank Dr. Leopold for his discussion.

There are two small points to bring out: First, that this material is even now quite precious. We were given it very generously by the Institute of Arthritis and Metabolic Diseases but have not been

able to obtain a new supply. We obviously have had to do our experiments on the material available first, that is, cortisone acetate and hydrocortisone free alcohol.

This is what we have done before either hydrolyzing or acetylating to get the other compounds.

This is a pinch-and-save type of experiment. As you noticed, the applicator is so designed that most of the applied material can be recovered after the experiment.

One thing which is quite curious about the discrepancy between the two types of experiments which Dr. Leopold's group and ours have done is, again, the apparently greater concentration of total corticoids in the aqueous after the local application by the Philadelphia group.

The thing which is particularly curious is that, on any theoretic basis, if one assumes that only the corticoid in solution is what penetrates and that the particles make no contribution to the penetration, one would expect a higher amount of penetration from the continuous applicator as used by us than from the drops which are applied intermittently as in Dr. Leopold's experiments.

From the results seen here just the opposite is indicated unless the total discrepancy is supplied by endogenous steroids. There is a whole order of magnitude difference, although our concentrations are expressed in terms of the amount in the aqueous rather than the amount per cc. This introduces a factor of four times, since the rabbit aqueous is approximately a quarter of a cc.

The discrepancy is still a mystery to me; and we will just have to do some more experimentation to try to hash out this difference. It may be possible that the particles in the higher concentrations do contribute in some manner to the amount of steroids which penetrate, although, at this time, I myself do not see how that could be.

STUDIES ON THE CRYSTALLINE LENS

VI. Mitotic activity in the epithelia of lenses cultured in various media

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The technique of culturing the ocular lens affords the opportunity of studying the metabolism of the intact organ in an isolated system where both physical and chemical environments can be controlled. To draw conclusions from such culture experiments, which are applicable to the lens in situ, the lens should necessarily be maintained in a physiologically normal state. This latter requisite presumably could be fulfilled simply by simulating the physical conditions which prevail in the eye and by employing aqueous humor as a culture medium.

However, to control the chemical environment of the lens it is necessary not only that the composition of any medium used for culture be known, but that the constituents can be added or eliminated at will. Not only is the composition of aqueous humor not known completely, but individual constituents cannot readily be removed, so that the favorable characteristics of aqueous humor as a culture medium are more than offset by other factors. The alternative would seem to be a wholly synthetic medium.

Merriam and Kinsey[1] have designed an apparatus for lens culture which appears to duplicate adequately some of the more essential physical conditions affecting the lens in vivo, and have employed several relatively simple synthetic media for culture. To provide a medium which might more adequately meet the nutritional requirements of the lens, Kinsey and Wachtl[2] devised a solution which contained many of the known constituents of the aqueous humor and in addition a number of substances which are thought to be important metabolically.

The adequacy of these media was tested by measuring glucose consumption and lactic acid production by the lens,[1,2] by determining the relative concentrations of various

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organic phosphates and other metabolically important constituents in the lens at the termination of culture, and by measuring the turnover rate and steady-state concentration of sodium between the lens and the medium.[2] Lenses cultured in the medium used by Merriam and Kinsey were found to utilize glucose and produce lactic acid in apparently normal amounts for periods of about a week; however, they gradually lost transparency, and the concentrations of glutathione and ascorbic acid decreased as they do in cataractous lenses in vivo. Lenses cultured for several days in the medium, devised by Kinsey and Wachtl, referred to in this paper as KEI medium, with plasma, were found to maintain approximately normal concentrations of glutathione and ascorbic acid, and the same concentrations and distribution of organic phosphates. They retained also a normal turnover rate and steady-state distribution of sodium between lens and medium.

It is known that some cellular processes show signs of impairment prior to discernible chemical changes. Kinsey and Grant[3] determined the relationship between the rate of cell division and oxygen uptake of yeast cells exposed to various concentrations of mustard gas. They found a normal oxygen uptake at concentrations of mustard gas which depressed cell division by 50 percent. Willmer[4] could find no definite correlation between carbohydrate utilization or lactic acid production and mitotic activity in cultures of chick fibroblasts. These observations suggested that the number of dividing cells might be a more sensitive index of the adequacy of a medium for lens culture than chemical changes. The present investigation is concerned with the evaluation of several synthetic media, and media from natural sources, on the basis of their ability to maintain normal mitosis in the epithelia of cultured lenses.

Methods

The apparatus and conditions employed for culture of lenses were essentially those described in a previous publication.[1] In the present experiments eight individual culture tubes, each containing one lens, were attached to the arm of a mechanical rocking device. The tubes were rocked gently at 12 times per minute. Temperature was maintained constant at 37°C. A gas mixture consisting of seven-percent oxygen, five-percent carbon dioxide, and 88-percent nitrogen was passed through a sintered glass filter to remove bacteria, bubbled through water to saturate it with moisture, and led into each tube. The flow was adjusted to approximately one liter per minute. S-shaped bubbler caps containing water were attached to the gas-exit arms of the culture tubes so that any interruption in the flow of gas could be noted visually. The pH of the medium was determined electrometrically before and after culture in a special culture tube modified to hold a glass electrode. In both instances the medium was equilibrated with the gas mixture until constant pH was obtained.

Lenses from the eyes of albino rabbits who weighed from 1.5 to 3.0 kg. were used for all the experiments. The animals were sacrificed by air embolism and the eyes immediately enucleated. The lenses were excised, extreme care being taken to avoid puncture of the capsule. One lens of each animal was fixed immediately to serve as a control. The other lens was placed epithelium up in a culture tube containing 5.0 ml. of medium which had been equilibrated for approximately two hours with the gas mixture to insure proper temperature and pH at the start of the experiment. The average time interval between the killing of the animal and introduction of the lens into the medium was about three minutes.

Culture periods varied from two to 48 hours. The longest period of culture without replacement of the medium was 24 hours. Sterile apparatus and technique were employed in all experiments exceeding nine hours, in which instance 0.1 ml. of an aqueous solution containing 200 units of potas-

sium penicillin, 0.5 mg. of dehydrostreptomycin sulfate, and 0.001 mg. of n-butylparahydroxybenzoate was added to each 5.0 ml. of medium.

Additional sodium bicarbonate was added to the rabbit plasma ultrafiltrate, the rabbit serum ultrafiltrate, and the TC medium 199 to maintain pH within narrow limits (7.4 to 7.6) throughout the experiments.

Flat mounts of epithelia of cultured lenses and control lenses were prepared to count the number of cells in mitosis. The technique used was that developed by von Sallmann.[8]* Lenses were fixed in Carnoy solution containing three parts of 95-percent ethyl alcohol and one part of glacial acetic acid. They were stained with Feulgen stain and the epithelia were mounted flat on microscope slides.

To facilitate counting each epithelium was cut with a razor blade into six wedge-shaped pieces. The number of cells in each of various phases of mitosis in each wedge was counted microscopically under oil at a magnification of 950 times. The total number of cells in each epithelium in all phases of mitosis was determined and expressed as percentage of cells in division in vitro with respect to that in vivo.

Media

The following media were tested for their adequacy as a culture medium:

1. KEI
2. KEI preparation, with 0.8-percent rabbit plasma
3. Inorganic salt solution, with glucose
4. TC 199
5. Serum ultrafiltrate (rabbit)
6. Plasma ultrafiltrate (rabbit)
7. Aqueous humor (beef)
8. Vitreous humor (rabbit)
9. Aqueous humor (rabbit)

The composition of the KEI medium is shown in column 2 of Table 1. The medium was prepared by mixing solutions in which the inorganic, the organic, and those constituents which were thought to be labile

TABLE 1

Preparation and composition of KEI medium*

	Mg./liter H_2O	Mg./liter Medium
Inorganic Solution		
Sodium chloride	15,350	6,140
Sodium bicarbonate	7,450	2,980
Calcium chloride 2 H_2O	510	204
Magnesium sulfate 7 H_2O	309	124
Dibasic sodium phosphate 7 H_2O	469	188
Zinc chloride	0.1	0.04
Sodium citrate	18	7
Sodium carbonate	790	316
Ferrous sulfate 7 H_2O	6.3	2.5
Cupric sulfate 5 H_2O	2.5	1.0
Potassium iodide	0.7	0.3
Manganous sulfate H_2O	2.5	1
Monobasic potassium phosphate	20	8
Organic Solution		
Glucose	6,250	1,250
Creatine H_2O	166	33
Ammonium sulfate	10	2
Biotin	0.5	0.1
Pyridoxine hydrochloride	6	1.2
Calcium pantothenate	5	1
Niacin	5	1
Folic acid	0.5	0.1
Cytochrome C	1.5	0.3
Inositol	0.5	0.1
Vitamin B_{12}	0.015	0.003
P-aminobenzoic acid	1	0.2
Choline chloride	500	100
L-leucine	285	57
L-isoleucine	285	57
L-phenylalanine	375	75
L-tyrosine	455	91
L-proline	335	67
L-hydroxyproline	385	77
L-alanine	185	37
L-glutamic acid	525	105
L-threonine	190	38
L-aspartic acid	355	71
L-serine	265	53
L-arginine monohydrochloride	400	80
L-lysine monohydrochloride	400	80
Glycine	185	37
L-histidine monochloride	400	80
L-tryptophan	350	70
L-valine	190	38
L-methionine	230	46
Potassium carbonate	1,630	326
Adenosine	25	5
Cytidine	25	5
Guanosine	25	5
Iosine	25	5
Uridine	25	5
Thymidine	25	5
Labile Solution	Mg./100 ml. H_2O	
Thiamine hydrochloride	0.5	1
Riboflavin	0.5	1
Adenosinetriphosphate disodium salt	2	4
Lactic acid 85%	(0.461 ml)	(0.922 ml)
Ascorbic acid	188	376
L-cysteine monohydrochloride	20	40
Sodium pyruvate	38	76
Alpha-tocopherol acetate	0.6	1.2
Coenzyme A	1	2
Diphosphopyridine nucleotide	1	2
Alpha-ketoglutaric acid	2	4
Succinic acid	2	4

* The three solutions and distilled water are mixed in the following proportion:

Inorganic solution	2 parts
Organic solution	1 part
Labile solution	1 part
Distilled water	1 part

* Dr. von Sallmann kindly instructed us in this technique.

TABLE 2

MITOTIC COUNTS IN LENS EPITHELIA AFTER CULTURE EXPRESSED AS PERCENTAGE OF THE CONTROL EYES

Lens	Medium	Hr. in Culture	Vitro/vivo (in percent)
32	KEI	6	52
33		6	38
34		6	79
35		6	56
44		6	78
45		6	22
B		9	86
C		9	57
D		9	34
6	KEI with 0.8% plasma	2	5
7		2	1
8		2	13
L_4		6	42
L_5		6	11
11		6	12
12		6	8
13		6	16
36		6	17
37		6	5
46		6	8
3		9	34
4		9	16
5		9	10
L_1		24	25
L_2		24	7
L_3		24	14
L_7		48	7
L_8		48	11
18	Inorganic plus 125 mg.% glucose	6	47
19		6	30
20		6	30
22		6	44
23		6	18
40	TC 199	6	101
41		6	105
42		6	83
43		6	145
55		6	87
56		6	100
60		24	8
61		24	32
62		24	15
57		48	19
58		48	12
59		48	5
48	Rabbit serum ultrafiltrate	6	15
49		6	27
50		6	41
51		6	17
28	Rabbit plasma ultrafiltrate	6	0
29		6	0
30		6	0
31		6	0
79	Rabbit vitreous humor	9	12
80		9	72
24	Beef aqueous humor	6	70
25		6	47
26		6	47
27		6	48
14	Rabbit aqueous humor	9	123
15		9	77
16		9	111
17		9	143
67		9	82
63		18	38
65		18	40
66		18	24
70		18	29

were dissolved separately in amounts shown in column 1, Table 1, and in volumes indicated at the bottom of Table 1.

The inorganic salt medium contained salts in the same concentrations as the KEI medium, and in addition 125 mg. percent of glucose.

TC medium 199 is synthetic and was devised by Parker[6] and prepared by Difco Laboratories, Detroit, Michigan. Rabbit serum ultrafiltrate was purchased from Microbiological Associates, Inc., Washington, D.C. Rabbit plasma ultrafiltrate was prepared by pressure filtration through cellophane. Beef aqueous humor was removed from cattle eyes within 15 to 30 minutes after death of the animals, frozen immediately, and stored in this state until used. Vitreous humor was collected from rabbit eyes immediately after enucleation, frozen immediately, and stored until used. Rabbit aqueous humor was obtained from topically anesthetized eyes by paracentesis. Precautions were taken to maintain conditions as sterile as possible throughout the procedure.

RESULTS

The number of epithelial cells in mitosis in the epithelium of each cultured lens is shown in Table 2 expressed as percentage of the number of cells in mitosis in the corresponding control lens. The average percentage for each condition of culture is shown in Table 3.

TABLE 3

AVERAGE MITOTIC COUNTS IN LENS EPITHELIA AFTER CULTURE EXPRESSED AS PERCENTAGE OF THE CONTROL EYES

Medium	Cultured Time in Hours 2	6	9	18	24	48
KEI	—	54	59	—	—	—
KEI with 0.8% plasma	6	15	20	—	15	9
Inorganic plus 125 mg. % glucose	—	34	—	—	—	—
TC 199	—	103	—	—	18	12
Rabbit serum ultrafiltrate	—	25	—	—	—	—
Rabbit plasma ultrafiltrate	—	0	—	—	—	—
Rabbit vitreous humor	—	—	42	—	—	—
Beef aqueous humor	—	53	—	—	—	—
Rabbit aqueous humor	—	—	107	33	—	—

The mitotic counts of lens epithelia cultured in KEI medium for six and nine hours was reduced to approximately half that of the uncultured control lenses. The addition of plasma to the KEI medium resulted in a decrease in the number of cells in mitosis to one-tenth to one-fifth normal. The period of culture did not appear to influence the results appreciably. The inorganic constituents of the KEI medium (with added glucose) alone could support approximately one-third of the normal number of cells in mitosis for six to nine hours.

The synthetic medium TC 199 maintained mitosis at the in vivo level for periods up to six hours; however, the nuclei of the cells showed signs of degeneration. After 24 or 48 hours of culture the number of cells in mitosis decreased to about one-seventh the number in vivo.

Neither rabbit serum ultrafiltrate nor rabbit plasma ultrafiltrate maintained a normal number of cells in mitosis. In the former about one-fourth the number of cells remained in a state of mitosis and in the latter no dividing cells were observed after six hours of culture.

Beef aqueous humor and rabbit vitreous humor were about equally effective in maintaining cell division. In both instances about one-half the cells continued to divide after six or nine hours of culture.

The number of cells in mitosis in the epithelia of lenses cultured for nine hours in rabbit aqueous humor was indistinguishable from that of uncultured control lenses, and the nuclei of the cells retained a normal appearance. After 18 hours of culture, however, the number of cells in mitosis decreased to an average of one third of the control lenses.

DISCUSSION

The use of the number of cells in mitosis as an index of adequacy of culture media appears to be a much more sensitive index than changes in various aspects of carbohydrate metabolism or even sodium distribution between lens and environment. This follows from the observation that significant depression of the number of epithelial cells in mitosis occurred after culture in media which maintained the lens in an apparently normal biochemical state.

The physical conditions of culture, including procedures employed in removing the lens from the eye and introducing it into the culture tube, must be without deleterious effect on cell division and nuclear development since the number of epithelial cells in mitosis and the appearance of the nuclei of lenses cultured for nine hours in aqueous humor were indistinguishable from uncultured control lenses. Thus, any depression of mitosis or tendency for nuclei to degenerate must result from a nonphysiologic chemical environment.

Among the various possible inadequacies the most likely would seem to be that the media may contain some toxic substance or that they may lack some essential nutrient, or both. With an exception to be noted below it is not feasible to distinguish with any degree of certainty between the several possibilities so far as a particular medium is concerned. From the standpoint of planning future experiments, however, it seems profitable to speculate concerning the relative role played by toxicity and deficiency with regard to interpreting the results obtained with the two most promising synthetic media.

In TC medium 199, the number of epithelial cells in mitosis remained normal for six hours of culture, but the nuclei showed

signs of degeneration. This finding suggests that the medium contains all of the substances necessary for mitosis but also contains a toxic constituent which causes the nuclei to degenerate. In KEI medium, the nuclei retained a normal appearance, but the number of cells in mitosis decreased. This suggests that there are no toxic substances present in KEI medium so far as nuclear development is concerned, but that it lacks one or more substances necessary for cell division, or alternatively contains substances inhibiting to mitosis.

That toxic compounds exist in plasma seems probable from the observation that the number of cells undergoing mitosis, after nine hours of culture, decreased significantly upon addition of plasma to the KEI medium.

While rabbit aqueous humor supported mitosis for nine hours it was observed that by the end of 18 hours of culture the number of dividing cells decreased to one third of normal. Again the question arises as to whether the depressed mitosis is the result of toxicity, in this instance accumulation of toxic compounds, or whether some substance and substances became depleted in the medium or lens or both. To investigate this problem two further experiments were performed. In the first, a lens was cultured for nine hours in one batch of aqueous humor and then replaced by a fresh lens which was cultured in the same batch of medium for an additional nine hours. The converse arrangement of culture conditions was employed in the second experiment, that is, at the end of nine hours of culture the aqueous humor was replaced by fresh aqueous humor and culture of the original lens was continued for another nine hours. No depression of mitosis was noted in either the lens cultured in medium previously used for nine hours or the lens cultured for 18 hours in the two separate lots of aqueous humor (tables 4 and 5).

The results from the first experiment would make it seem unlikely that the depression of mitosis which occurs between nine and 18 hours of culture in the same medium, results from the toxic effect of accumulated end products of metabolism. The second experiment, which shows that the addition of fresh aqueous humor at the end of nine hours of culture enables the epithelium to maintain a normal number of cells in mitosis, suggests that mitosis is depressed after nine hours because of the depletion of some essential substance ôr substances from the medium. However, again considering the first experiment, it is evident that even though the medium after nine hours has become partially depleted, a fresh lens contains sufficient reserves to maintain mitosis at a normal rate. In other words, it appears that both the medium and the lens become partially depleted of those substances necessary for the maintenance of cell divi-

TABLE 4

MITOTIC COUNTS IN LENS EPITHELIA AFTER CULTURE EXPRESSED AS PERCENTAGE OF THE CONTROL EYES

Lens	Medium	Hr. in Culture	Vitro/vivo (in percent)
	Rabbit aqueous humor:		
71	Previously used for	9	92
72	culturing another	9	99
73	lens for 9 hours	9	103
68	Medium renewed af-	18	125
69	ter 9 hours	18	92
74	Solution A added af-	18	61
75	ter 9 hours	18	8
76	Solutions A and B	18	47
77	added after 9 hours	18	29

TABLE 5

AVERAGE MITOTIC COUNTS IN LENS EPITHELIA AFTER CULTURE EXPRESSED AS PERCENTAGE OF THE CONTROL EYES

Medium	Culture Time in Hours	
	9	18
Rabbit aqueous humor		
Previously used for culturing another lens for 9 hours	98	—
Medium renewed after 9 hours	—	108
Solution A added after 9 hours	—	34
Solutions A and B added after 9 hours	—	38

TABLE 6

PREPARATION AND COMPOSITION OF SUPPLEMENT SOLUTIONS A AND B*

Solution A	Mg./100 ml. H_2O	*Solution B*	Mg./10 ml. H_2O
Biotin	0.5	Thiamine HCl	0.5
Pyridoxine HCl	6.0	Riboflavin	0.5
Ca-pantothenate	5.0	Adenosinetriphosphate disodium salt	2.0
Nicotinic acid	5.0	Ascorbic acid	188.0
Folic acid	0.5	Sodium pyruvate	38.0
Cytochrome C	0.5	Coenzyme A	1.0
Inositol	0.5	Diphosphopyridine nucleotide	1.0
Vitamin B_{12}	0.015	Alpha-ketoglutaric acid	2.0
Choline HCl	500.0	Succinic acid	2.0
P-aminobenzoic acid	1.0	L-cysteine HCl	20.0

* 0.1 ml. of supplement solution was added to 5.0 ml. of medium after 9 hours of culture.

sion during nine hours of culture. Whether depletion occurs primarily because of utilization of nutrients by the lens or in part because of their chemical lability cannot be determined from the present experiments. In an attempt to identify the compounds involved, and thus perhaps gain some insight into the nature of some compounds which seem essential for mitosis, several substances (table 6) which might be expected to be present initially in the aqueous humor in trace amounts, or to have disappeared because of chemical lability, were added to aqueous humor after nine hours of culture.

Fortification of the aqueous humor after nine hours of culture with solution A, or solutions A and B did not result in the maintenance of a normal number of cells in mitosis (tables 4 and 5). The identity of those substances which become depleted thus remains unknown.

SUMMARY

The ability to maintain normal mitosis in epithelial cells of cultured lenses has been used as a measure of the adequacy of various culture media.

The degree of effectiveness of the nine media tested in maintaining cells in mitosis following six to nine hours of culture is shown below. The figures in parentheses represent the percentage of the average number of epithelial cells in mitosis of the cultured lens divided by the number in the control lens.

Rabbit aqueous humor (107)
TC medium 199 (103)
KEI medium (57)
Beef aqueous humor (53)
Rabbit vitreous humor (42)
Inorganic constituents of KEI medium plus 125 mg. percent glucose (34)
Rabbit serum ultrafiltrate (25)
KEI medium with plasma (17)
Rabbit plasma ultrafiltrate (0)

Rabbit aqueous humor and TC medium 199 both maintained a normal mitotic count but the nuclei of the cells in the synthetic medium showed signs of degeneration. The nuclei in the KEI synthetic medium were normal in appearance.

In general, extension of the period of culture reduced the number of cells in mitosis. Substitution of fresh aqueous humor, or a freshly renewed lens after nine hours of culture in aqueous humor, prevented the decrease in the number of dividing cells. It is concluded that some essential substance is depleted in both the medium and in the lens during the course of the first nine hours of culture. The identity of this substance or substances could not be established.

The use of the number of cells in mitosis appears to afford a much more sensitive index of the adequacy of a medium for lens culture than deviation from normal of the levels of various chemical constituents of the lens.

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Discussion

Dr. H. L. Ormsby (Toronto, Canada): I wish to congratulate the authors on their rather unique approach to the problem of in vitro cultivation of whole lenses. The use of mitotic activity would appear to offer a sensitive method for ascertaining the adequacy of various synthetic media in supporting the metabolism of the lens.

Those of us who have watched the developments in the tissue-culture field will appreciate the problems still to be worked out in the development of synthetic media. These problems are essentially those of finding, by the process of addition and subtraction, a complete supportive media for the growth of an individual tissue. Initially, tissue-culture methods depended on complex components such as serum and tissue extracts; and, even with the improvement of synthetic media, it is still necessary, in most instances, to add varying amounts of these extracts.

It seems appropriate for research teams working on physiologic problems of the eye to apply the present techniques in the studies of synthetic media to the metabolism and growth of ocular tissue. This is obviously a difficult and complex problem but, in time, it is almost certain that a synthetic medium will be found which will support the metabolism of the lens. When this occurs, the way will be open, by varying the media, to a further understanding of the chemical changes involved in the metabolism of normal lenses and in pathologic states.

Dr. John E. Harris (Portland, Oregon): I certainly would agree with Dr. Ormsby that this is an excellent presentation. The question of when a cell is living or dead is not always easy to answer. The authors have undoubtedly chosen a most rigorous criterion by which to determine the efficacy of their culture media.

I was wondering whether you might have tried graded oxygen levels. I was thinking of some experiments on tissue culture, by Osgood and his associates, employing what they term a "gradient culture" of leukemic leukocytes. In effect they suspend cells in a tube of media and find that they obtain best reproduction at a definite critical level below the surface. This may be due to a critical oxygen tension or to some other more subtle influence still undetermined.

Dr. Carl Wachtl (in closing): I would like to thank Dr. Ormsby for discussing the paper.

In answer to Dr. Harris, we use a relatively low oxygen level, seven percent, which will give rise to a concentration in the medium similar to that found by Dr. Friedenwald in aqueous humor.

In our system, we cannot determine oxygen uptake; but we can determine directly metabolic activities by analysis of different intermediates in the medium following culture.

For instance, it is known that some glycolysis takes place; but the path of metabolism beyond that is not quite certain. Some think the citric acid cycle is followed; and some think the path is through phosphogluconic acid, ribulose phosphate, and so forth. This question would need clarification.

ASCORBIC-DEHYDROASCORBIC ACID AS AN OXIDATION-REDUCTION SYSTEM*

In the maintenance of the metabolism of lens cultured in vitro

I. Weight and cation equilibrium

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Introduction

In order for a tissue to be an efficient transmitter of light, it must be homogeneous with respect to maintaining a constant index of refraction. This can be achieved by having cells of the same index of refraction arranged parallel, by maintaining minimal space between these cells, and excluding substances such as hemoglobin which would interfere with the transmission of light. The latter two requirements can be obtained with minimal extracellular space and avascularity.

The tissues that compose an optical system require an active metabolism to maintain themselves and regulate their intracellular and extracellular space relationships. If this metabolism is partly respiratory in nature then it is assumed to contain hydrogen transport systems. Oxygen as an hydrogen acceptor must then be available in the surrounding media in sufficient amounts to meet the respiratory requirements of the tissues. However, it is conceivable that in an avascular environment other substances may act as hydrogen acceptors.

With these considerations in mind, one can turn to the eye where tissues with optical properties exist. The cornea, lens, vitreous, and aqueous are transmitters of light. Associated with their transparency is a minimum extracellular space and an avascularity. Figure 1 illustrates the relative proportion of vascular and avascular mass in the eye.

The cornea and lens are well known to have an active metabolism and recent work by Zeller (1955) has demonstrated anabolic enzymes in the vitreous.

The glycolytic activity of the lens and cornea has been well established experimentally (Bellows, 1944[a], Nordmann, 1954[a]). The question of respiration of these tissues has been considered in terms of oxygen uptake and carbon-dioxide production. But Christiansen and Leinfelder (1952) have recently thrown doubt on the value of oxygen uptake studies as indicative of respiration in the lens. They interpreted the oxygen uptake as nonenzymatic—the representation of auto-oxidation of reduced substances.

There is, however, other evidence indicating that elements of the respiratory cycle (or rather hydrogen transport system) occur in these tissues. Kinsey and Frohman (1951) have demonstrated the presence of cytochrome C and riboflavin in the lens epithelium. Herrmann and Hickman (1948)

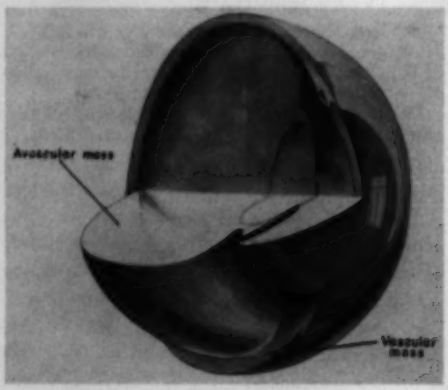

Fig. 1 (Schwartz and Leinfelder). Vascular and avascular mass in the eye.

* From the Department of Ophthalmology and Physiology, College of Medicine, State University of Iowa.

have indicated the presence of cytochrome and Bessey and Lowry (1944) identified riboflavin in the corneal epithelial layers. Ely (1951) has also shown the oxidation of members of the Krebs citric acid cycle by lens homogenates.

Further evidence for respiratory activity has been shown by in vitro and in vivo studies indicating the requirements of oxygen by lens and cornea to maintain physiologic steady states. Harris et al. (1954) demonstrated the aerobic dependence of the lens for reversing a cold-induced cation shift. Schwartz, Danes, and Leinfelder (1954) related weight maintenance (and presumably water content) in lens and cornea with the presence of oxygen. Davson (1955) has also demonstrated recently corneal dependence on oxygen in vitro in order to prevent hydration. Similar observations have been noted in vivo by Langham (1952) and by Smelser and Ozanics (1953) with regard to corneal hazing produced by contact lenses.

Associated with the avascularity and transparency of optical tissues is the presence of high concentrations of reduced substances which have been identified as ascorbic acid and reduced glutathione in the lens (Henkes, 1944) and cornea (Herrmann and Moses, 1945; Pirie, 1946). These substances are mainly concentrated in the superficial or epithelial layers. Only ascorbic acid is found in the aqueous (Nakamura, 1935) and vitreous (Johnson, 1936). There is usually found associated with these reduced substances their oxidized counterparts—dehydroascorbic acid and oxidized glutathione. They are present in smaller concentrations as identified in the lens (Podesta and Baucke, 1938) and aqueous (Langham, 1950, Kinsey, 1950).

Several theories have been advanced in order to explain the high level of aqueous ascorbic acid (Bellows, 1944[b]). One hypothesis has been the direct synthesis of ascorbic acid by the lens. This has largely found no experimental support.

Goldmann and Buschke (1935, 1936) proposed that ascorbic acid enters the eye from the blood in the oxidized form, dehydroascorbic acid, and is reduced to ascorbic acid by the lens. The ascorbic acid is then considered impermeable and cannot escape through the blood-aqueous barrier. Their conclusion in regard to the ability of the lens to reduce dehydroascorbic acid followed from the observation that in aphakic eyes the concentration of ascorbic acid is diminished while the dehydroascorbic acid level is raised.

Kinsey (1950) reinvestigated this problem and found no ascorbic acid formation after injection of dehydroascorbic acid in enucleated eyes and in vivo. However, the time used was physiologically small (in the order of six minutes) and the concentration of dehydroascorbic acid was much greater than physiologic levels. Langham (1950) confirmed again Goldmann and Buschke's observation of a decrease in the concentration of ascorbic acid and an increase in the concentration of dehydroascorbic acid in the aphakic eye.

The third hypothesis is that advanced by v. Eckelen et al. (1934) and Friedenwald et al. (1939, 1941, 1943) of the secretion of ascorbic acid by the ciliary epithelium. In support of this hypothesis are the in vivo experiments of Kinsey (1950) and Langham (1950) which indicate an active secretory process for aqueous ascorbic acid production whose level increases with plasma levels. However, there is a definite barrier against the diffusion of dehydroascorbic acid from blood to aqueous when the secretory mechanism for ascorbic acid is unsaturated with ascorbic acid. Blood dehydroascorbic acid is then converted into ascorbic acid and secreted as such into the aqueous. If the system is first saturated with ascorbic acid, however, then dehydroascorbic acid from the blood penetrates the barrier (Langham, 1950).

These observations indicate not only a secretory system for ascorbic acid, but also an active mechanism attempting to maintain

a constant level of dehydroascorbic acid in the aqueous, which is not necessarily correlated with ascorbic acid secretion.

As both ascorbic acid and dehydroascorbic acid exist in the aqueous they form a thermodynamically reversible oxidation-reduction system. A definite redox level exists in the aqueous, vitreous, and lens (about +100 m.v.) which Nordmann (1954[b]) believes is stabilized by ascorbic-dehydroascorbic acid.

It should also be noted that these substances exist in aqueous whose oxygen content (0.08 to 0.12 vol. percent, Friedenwald and Pierce, 1933) is about one hundredth the level of blood oxygen. The question arises as to whether this oxygen level is sufficient to supply the respiratory requirements of the tissues of the anterior chamber in their need for an hydrogen acceptor.

In view of the seeming paradox of the avascular optical tissues which need hydrogen acceptors for active respiratory metabolism under anoxic conditions and the fact that ascorbic acid and dehydroascorbic acid both exist in the aqueous, it is suggested that the problem should be reinvestigated with several additional criteria.

It is postulated that both dehydroascorbic acid and ascorbic acid are maintained in the aqueous at a definite ratio forming a steady state. Thus there exists a redox potential between these substances. Under the relatively anoxic conditions of the anterior chamber the respiratory system of the lens and cornea pass their hydrogen onto dehydroascorbic which thus acts as an hydrogen acceptor. This transport is best facilitated at the physiologic redox level. Both compounds are needed at a definite redox level under an anoxic environment (fig. 2).

In this way the respiratory metabolism of the ocular refractive media can be maintained by the use of dehydroascorbic acid as an hydrogen acceptor without involving an opaque respiratory pigment.

To obtain experimental evidence for this hypothesis the following experiments were performed. Lenses were maintained in vitro for 48 hours in artificial aqueous under anoxic conditions with added amounts of dehydroascorbic acid and/or ascorbic acid, so

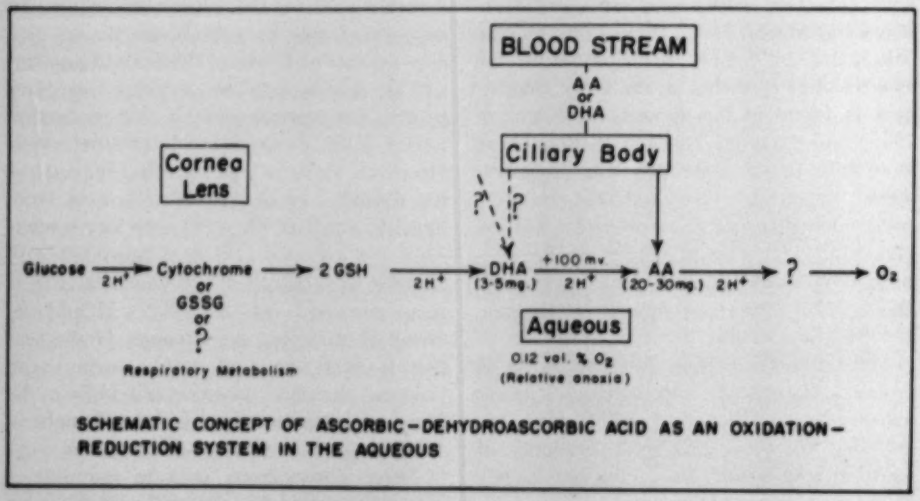

Fig. 2 (Schwartz and Leinfelder). Schematic concept of ascorbic-dehydroascorbic acid as an oxidation-reduction system in the aqueous.

that a definite redox potential was obtained. As ascorbic and dehydroascorbic acid are unstable due to their ease of oxidation, their effect had to be defined under anoxic conditions. Comparison of metabolic activity was then made by comparing these lenses with lenses under nitrogen and air atmospheres. pH was maintained relatively constant in the physiologic range. The redox potentials and pH values of the culture fluid were measured. The weight change of the lenses and sodium, potassium, glucose, lactic acid, ascorbic acid, and dehydroascorbic acid were measured in both fluids and lenses. This paper will limit itself to discussion of sodium, potassium, and weight changes.

Materials and Methods

Essentially the same technique as previously outlined (Schwartz, Danes, and Leinfelder, 1954) was followed.

An attempt was made to maintain a physiologic culture media by obtaining (1) asepsis, (2) solutions approximating aqueous regarding tonicity (152 milliosmoles), glucose (80 mg. percent), and salt content, (3) a physiologic pH range by the use of a bicarbonate buffer, and (4) constant temperature by incubation at 37.5°C.

The bovine lens was used for all studies. These were obtained four to five hours after enucleation from the abattoir* and arrived packed in ice.

The experimental solution used was an artificial aqueous solution devised by Merriam and Kinsey (1950). The lactic acid and urea ingredients however were omitted.†

The solution was prepared and autoclaved as required without sodium bicarbonate which was autoclaved separately. (Previously we had filtered the sodium bicarbonate through a Selas filter.) After cooling, the sodium bicarbonate was thoroughly gassed with 100-percent carbon dioxide for 20 minutes and then added in proper proportions to make up the final solution. One-hundred-percent carbon dioxide was then bubbled through this final solution for 10 minutes followed by five-percent carbon dioxide in air for approximately 20 minutes so that a pH of about 7.30 was obtained. Five-hundred cc. of this solution were then placed in 500-cc. flasks which were sealed with a rubber sleeve. The resultant pH obtained after equilibrium had taken place was about 7.40. A 500-cc. volume was used so that more than an adequate buffering system was provided for the lens metabolism.

The solutions were then incubated for four to five days. Any cloudiness of the media was considered as contamination and these solutions were discarded.

The use of a bicarbonate buffer and ascorbic acid and dehydroascorbic acid solutions required the anaerobic determination of pH of the solutions. This was done using a hypodermic electrode with a Beckman pH meter. Redox potentials were also measured using the Beckman model and a platinum wire electrode. Both the pH glass and platinum electrodes were placed in an air tight glass chamber of our own design, and solutions were then introduced with a hypodermic needle and syringe through a rubber sleeve (as illustrated in figure 3). Both pH and redox could thus be measured on the same sample anaerobically. It was determined that each electrode did not influence the reading of the other electrode, when one was disconnected out of the circuit. pH readings showed usually no more variation than $\pm$ 0.02 units while the redox values varied $\pm$ 10 millivolts.

Gassing tubes were designed to fit the mouth of the 500-cc. flasks. These tubes as diagramed (fig. 4) essentially consisted of a long glass entrance tube surrounded by an exit chamber. Gas could be introduced

* Courtesy of Wilson and Company, Cedar Rapids, Iowa.

† The constituents used are: Sodium chloride (0.90 percent) 100 ml.; potassium chloride (1.15 percent) 5.0 ml.; calcium chloride (1.22 percent) 1.0 ml.; magnesium sulfate hepta hydrate (3.82 percent) 0.6 ml.; monobasic potassium phosphate (2.11 percent) 1.0 ml.; sodium bicarbonate (1.30 percent) 42.0 ml.; dextrose (12.0 percent) 1.0 ml.; total, 150.6 ml.

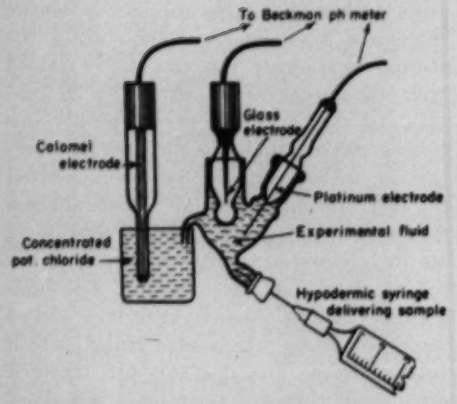

Fig. 3 (Schwartz and Leinfelder). Glass chamber for anaerobic pH and redox determinations.

through the rubber sleeve of the entrance tube with a hypodermic needle allowed to bubble through the solution and then escape via a hypodermic needle placed in the exit tube. A solution could thus be thoroughly gassed.

The lenses were handled with aseptic technique; the greatest possible precautions were taken to maintain sterility. They were removed intact by an anterior approach. Lenses with ruptured or punctured capsules were discarded. After removal the lens was blotted dry and weighed on a torsion balance with an accuracy of ± 2.0 mg. Each lens was then placed in the 500 cc. incubated solutions and a rubber sleeve or a gassing tube used to seal the mouth of the flask if gassing was required. The glass-rubber connections of the gassing tubes (as an extra precaution against leaks) were sealed with paraffin.

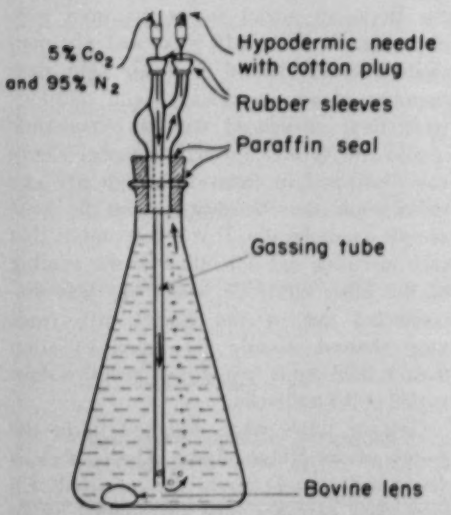

Fig. 4 (Schwartz and Leinfelder). Gassing flask for in vitro culture of bovine lens.

The flasks with lenses were then incubated at 37.5°C. for a period of five to six hours. As these lenses arrived packed in ice (about 7°C.) they gained weight with this low temperature (Schwartz, Danes, and Leinfelder, 1954) and underwent a cation shift (Harris, 1953). To partially offset this shift, an equilibrium period of five to six hours at 37.5°C. was used.

The solutions were then gassed with five-percent carbon dioxide and 95-percent nitrogen for 15 minutes. The gas was passed over hot copper pellets in a quartz tube in order to remove all residual oxygen.

L-dehydroascorbic and/or L-ascorbic acid* were then added to these solutions. These compounds were kept at 4°C. in sealed bottles, being opened just prior to use. Physiologic concentrations of ascorbic and dehydroascorbic acid were used, in amounts of 20 mg. percent for ascorbic (except in one experiment where 30 mg. percent was used) and 5.0 mg. percent for dehydroascorbic. Each substance was added separately (the ascorbic acid after the dehydroascorbic acid) by making up the desired concentrations with "nitrogenized" artificial aqueous in a syringe and injecting into the flasks through a Swinny filter to obtain a sterile solution. It was noted that ascorbic acid dissolved readily in the solution but dehydroascorbic acid dissolved poorly.

The glass-rubber connections were then resealed with paraffin and the level of the fluid in the central gassing tube marked.

* Nutritional Biochemical Company, Cleveland, Ohio.

The solutions were then returned to the incubator.

After approximately 48 hours observations were made. The fluid level in the central gassing tube was noted as well as the color of the solutions and which surface of the lens rested on the bottom of the flask. Any opacities or changes in transparency of the lens were recorded.

Samples of fluid were then withdrawn from the flasks through the rubber sleeves with a hypodermic syringe and injected into the anaerobic pH and redox chamber, thus allowing minimal contact with air. pH and redox values were then recorded at room temperature. Fluid samples for biochemical determination were withdrawn anaerobically and aerobically. The anaerobic samples were delivered into containers under oil. All substances for biochemical analysis contained 2.0 ml. of a three-percent sodium fluoride solution. The lenses were then removed, dried, and weighed, and placed in usually 30 cc. of the fluid sample under paraffin oil. The samples were then stored at −20°C.

Blanks (flasks) with no tissues present as well as controls in all experiments were run.

Solutions that appeared cloudy at the end of the experimental period were considered contaminated and discarded.

Sodium and potassium were measured using the Baird flame photometer. Sodium and potassium values were calculated as milliequivalents per liter for the fluids and milliequivalents per 100 gm. for lens-corrections being made for dilution factors. Weight changes are expressed as percent gain of initial weight.

Observations

The data regarding the pH and redox determinations are presented in Table 1.

The means of the pH varied from 7.34 to 7.58 while the means of the redox values varied from +137 to −83.

The solutions gassed with five-percent carbon dioxide and nitrogen had the highest pH while the ascorbic acid were the lowest. However, the important consideration is that although the pH fluctuated in what we consider a physiologic range, the redox varied widely with the experimental conditions.

Thus when ascorbic acid was used alone a negative redox value was obtained. Dehydroascorbic acid alone provided a low positive value. A combination of these substances gave a negative value between dehydroascorbic acid and ascorbic acid. An air atmosphere gave a high positive value while the nitrogen atmosphere redox was between air and dehydroascorbic acid. Thus a range of redox values was obtained fairly readily while maintaining a relatively physiologic pH.

It will be noted that with these experiments we were unable to obtain, with the concentrations used of ascorbic-dehydroascorbic acid, a redox level of about +100 millivolts which would simulate the aqueous level.

Gross observations of the flasks and the lenses provided the following findings:

1. The surface of the lens that rested on the bottom of the flask usually by the end of 48 hours showed a small central opacity, grayish-white in nature and apparently confined to the capsular surface. Otherwise no significant changes or opacities were noted in the lens.

2. Small multiple gas bubbles were noted forming a ring at the equator of the lens and/or covering its posterior surface in many experiments. They occurred particularly in the dehydroascorbic acid-ascorbic acid experiments, although a few were noted in the ascorbic acid or dehydroascorbic acid flasks.

3. The fluid level in the gassing tubes was usually high in the flasks containing ascorbic acid or ascorbic-dehydroascorbic acid. If we consider the flasks as sealed tight, then the raised level is probably an expression of increased gas pressure within the system. This may be due to generation of carbon-dioxide from the bicarbonate medium through the action of the ascorbic acid.

TABLE 1

REDOX AND pH VALUES UNDER VARIED EXPERIMENTAL CONDITIONS

	5% CO_2 in Air				5% CO_2 & 95% N_2				DHA 5 mg% 5% CO_2 & 95% N_2				AA 20 mg% DHA 5 mg% & 5% CO_2 & 95% N_2				AA 20 & 30 mg% 5% CO_2 & 95% N_2			
	Control Fluids		Lenses†		Control Fluids		Lenses†		Control Fluids		Lenses†		Control Fluids		Lenses†		Control Fluids		Lenses†	
Final:	pH	redox*	pH	redox	pH	redox	pH	redox	pH	redox	pH	redox	pH	redox	pH	redox	pH	redox	pH	redox
ExpRo4	7.51	+172	7.48	+148	7.63	+140	7.49	+122									7.43	−103	7.38	−95
	7.49	+ 78	7.48	+110	7.58	+142	7.51	+ 84									7.45	− 76	7.39	−95
			7.41	+165			7.51	+105											7.28	−84
																			7.34	−78
ExpRo6	7.41	+182	7.38	+153	7.48	+130	7.52	+140	7.48	+20	7.45	+115								
	7.48	+192	7.38	+175	7.55	+166	7.47	+122	7.50	+52	7.55	+ 80								
			7.44	+172			7.52	+133			7.48	+ 75								
ExpRo8	7.47	+150	7.50	+150	7.66	+145	7.53	+148	7.61	+48	7.45	+ 32								
	7.50	+165	7.48	+ 98	7.65	+154	7.65	+115			7.49	+ 45								
											7.50	+ 40								
ExpRo9	7.40	+128	7.33	+110	7.68	+100	7.48	+ 68					7.38	−60	7.32	−24				
	7.38	+138	7.36	+133	7.58	+132	7.55	+112					7.35	−52	7.30	−42				
															7.37	−32				
															7.38	−34				
															7.33	−46				
															7.38	−38				
Mean	7.45	+171	7.42	+135	7.58	+133	7.56	+108	7.55	+36	7.50	+ 74	7.37	−56	7.34	−35	7.43	− 83	7.38	−69

* Redox expressed as millivolts.
† Values for fluids with lenses.

4. There was a definite color change in some of the solutions containing ascorbic acid, dehydroascorbic acid, or both, that increased with time. We had previously noted that ascorbic acid solutions under our experimental conditions with an air atmosphere turned dark brown in color, the intensity increasing with time. This was believed to be due to aerobic decomposition of the ascorbic acid. However, with the use of completely "nitrogenized" solutions this change was avoided except some minor color changes were noted as follows:

Experimental Variable	Number of Flasks	Color
Ascorbic-acid solutions	7	Clear
	2	Yellowish tinge
	1	Brownish tinge
Dehydroascorbic-acid solutions	2	Clear
	4	Yellowish tinge
	3	Brownish tinge
Ascorbic-dehydro-ascorbic acid solutions	7	Yellowish tinge
	1	Brownish tinge

No correlation was noted between the color of the solution and the pH or redox, the absence or presence of a lens, or with changes in the weight, sodium or potassium content of fluids or potassium content of the lenses.

The color reactions may represent some degenerations due to residual oxygen, or the anaerobic decomposition reported with dehydroascorbic acid solutions. (Rosenfield, 1943).

Weight change

Weight change as percent of initial weight of the lenses is plotted against the redox values. As each experimental condition, for example, ascorbic acid, dehydroascorbic acid, nitrogen, and so forth, occupied a range on the redox scale all the experimental values can be chartered on one graph as illustrated.

The initial weight of the lenses will be greater than the physiologic level due to the fact that they were measured immediately after the lenses were removed from enucleated eyes packed in ice. Their weight gain thus represents a cold-induced hydration. Recovery with loss of weight should occur with incubation temperature (Schwartz, Danes, and Leinfelder, 1954).

The plotted results (fig. 5) show a slight weight loss with an air atmosphere, little if any significant gain under nitrogen, but a definite weight loss with dehydroascorbic acid. Lenses in ascorbic and dehydroascorbic-ascorbic acid both showed increased weight gain.

Sodium/potassium ratios of fluid

Similarly the sodium/potassium ratios of the fluids (expressed in milli-equivalents per liter) in which the lenses were cultured were plotted against the redox values of the solutions.

Graphically (fig. 6) these results are expressed as the ratio between the experimental fluids and the average value of control fluids in which no lenses were placed.

Results indicate that the ratio decreases under nitrogen, rises again with dehydroascorbic acid, decreases slightly with dehydroascorbic-ascorbic acid, and shows a further decrease with ascorbic acid.

Absolute potassium content of lenses

The absolute calculated potassium content of the lenses (as milli-equivalents per 100 gm.) is charted against the redox value of the solutions (fig. 7).

The line parallel to the abscissa represents the average value of seven lenses determined after removal from the globe.

With a nitrogen atmosphere there appears to be a tendency for a decrease in potassium content. Dehydroascorbic acid and dehydroascorbic-ascorbic acid solutions tend to slightly lower potassium levels below average values. With ascorbic acid there is a definite lowering effect.

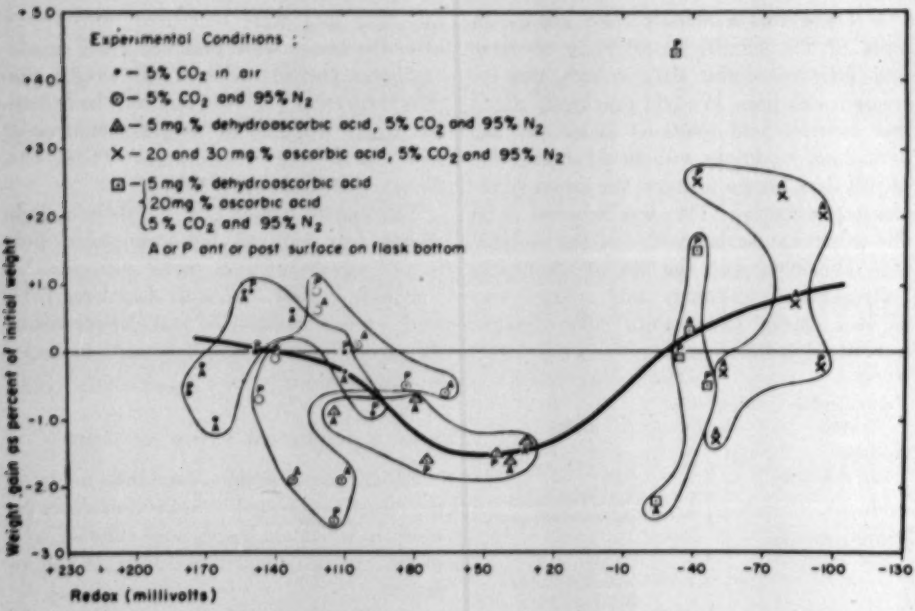

Fig. 5 (Schwartz and Leinfelder). Relationship between weight change of lens and redox level of solutions.

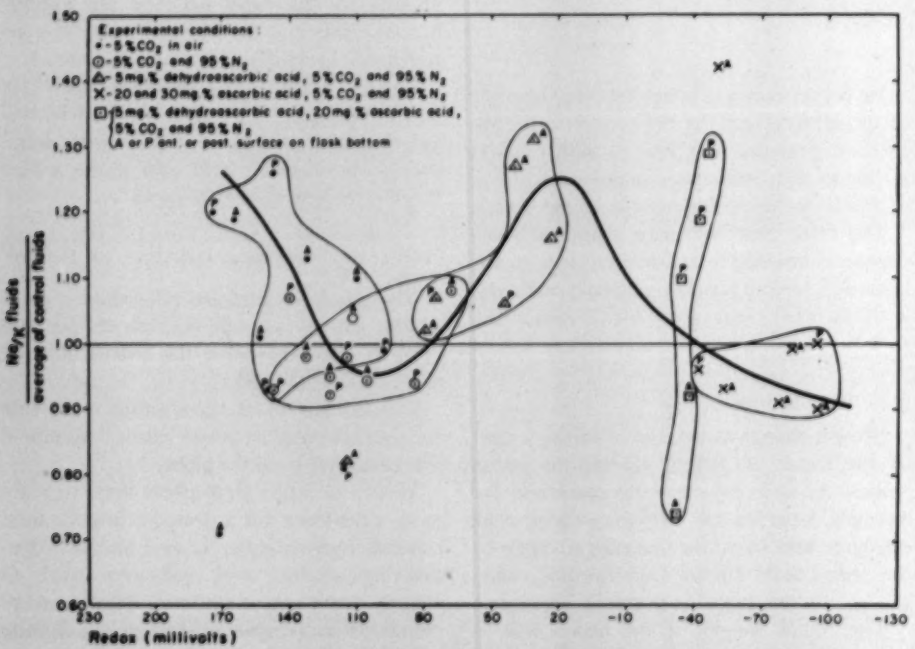

Fig. 6 (Schwartz and Leinfelder). Relationships between sodium/potassium ratio and redox level of solutions.

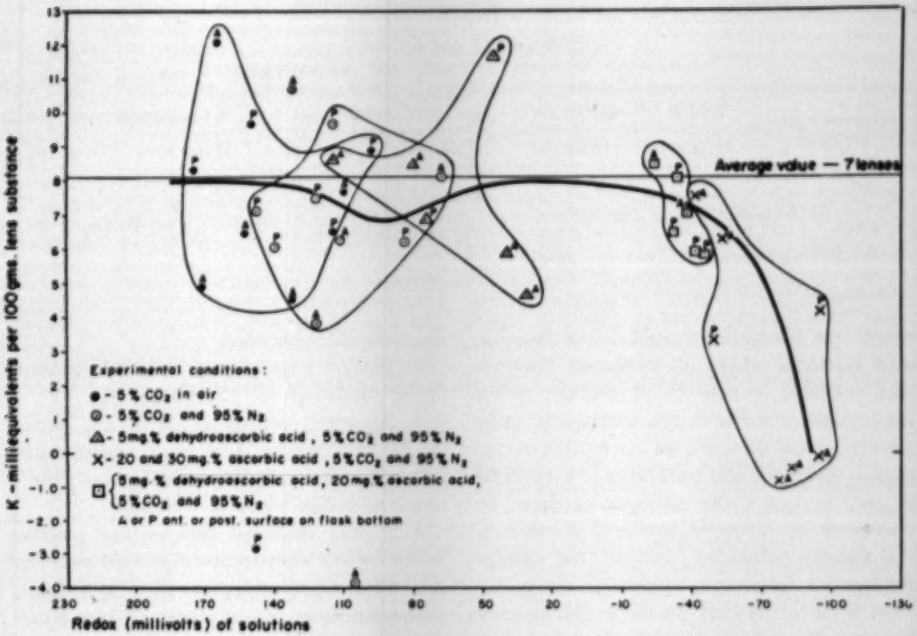
K - milliequivalents per 100 gms. lens substance
Redox (millivolts) of solutions
Average value — 7 lenses
Experimental conditions:
● - 5% CO2 in air
⊙ - 5% CO2 and 95% N2
△ - 5mg.% dehydroascorbic acid, 5% CO2 and 95% N2
X - 20 and 30 mg.% ascorbic acid, 5% CO2 and 95% N2
□ 5mg.% dehydroascorbic acid, 20mg.% ascorbic acid, 5% CO2 and 95% N2
A or P ant. or post. surface on flask bottom

Fig. 7 (Schwartz and Leinfelder). Relationship between potassium content of lenses and redox level of solutions.

Discussion

The proper evaluation of the proposed hypothesis required a variation of redox potential under conditions of anoxia and physiologic pH. It was hoped in this way that dehydroascorbic-ascorbic acid could thus be shown to maintain physiologic function, such as weight and cation equilibrium. Ascorbic acid and dehydroascorbic acid were used alone in order to evaluate their individual effects.

The results indicate that the pH range was maintained within physiologic limits while a definite variation of redox potential was obtained.

Relative anoxia was maintained as shown by the absence of marked "browning" reactions of the ascorbic and dehydroascorbic acid solutions. Although some minor color changes were observed these may be due to residual oxygen or anaerobic decomposition.

One other factor entering into the interpretation of the observations was the variable of which surface of the lens rested on the flask bottom. This factor would be considered important especially if the anterior surface of the lens rested downward. Pressure changes through the capsule on the epithelium and poor exposure to the solution may affect the water content and cation exchange if the epithelium is considered as the source of adenosinephosphates for the lens (Frohman and Kinsey, 1952).

A major consideration of the hypothesis is the redox level at which hydrogen transport optimally occurs. Extremes of potential would theoretically inhibit this transport and affect lens metabolism. Thus it could be argued (table 2) that at high positive or negative potentials weight gain, decrease in sodium/potassium ratios of the culture fluid, and loss of potassium from the lenses would occur. The changes in the cation equilibrium and hydration would then express a metabolic defect. These changes were seen to occur readily under our experimental condi-

TABLE 2

SUMMARY OF OBSERVATIONS

A COMPARISON BETWEEN HYPOTHETIC AND OBSERVED RESULTS

Experimental Variable	Weight Change of Lens		Na/K of Solutions		K Concentration of Lens	
	Hypothetic	Observed	Hypothetic	Observed	Hypothetic	Observed
Air	−	±	+	+	±	±
N_2	+	±	−	−	−	−
DHA	−	−	+	+	+	±
AA-DHA	+	±	−	±	−	−
AA	+	+	−	−	−	−

tions with nitrogen atmosphere and ascorbic acid solutions where no hydrogen acceptor was available. When the physiologic redox level was reached with dehydroascorbic acid (which could then act as an hydrogen acceptor under anoxic conditions), a reversal of the changes under nitrogen occurred as evidenced by a weight loss and increase in the sodium/potassium ratio of the culture fluid and a relative steadiness of the potassium level in the lens. In these experiments however the "ideal" physiologic redox level using ascorbic-dehydroascorbic acid was not obtained.

Because of the biologic variability inherent in the use of many lenses the results must be interpreted in terms of trends or qualitative data.

Experiments are in progress to continue these studies in terms of ascorbic acid-dehydroascorbic acid concentrations with perfusion on a single lens in vitro.

SUMMARY

1. An hypothesis is outlined whereby ascorbic-dehydroascorbic acid acting as an oxidation-reduction system serves to transport hydrogen from the ocular avascular respiratory metabolic tissues. It is proposed that both these substances are maintained at steady state ratios in the anoxic aqueous at a definite redox level.

2. Bovine lenses were cultured in vitro under conditions of physiologic pH.

3. Ascorbic, dehydroascrobic, and ascorbic-dehydroascorbic acid were added to the culture under anoxic conditions so that a range of redox levels was obtained.

4. It was observed that at low positive redox values dehydroascorbic acid reversed the effects of weight gain, shift in sodium/potassium ratio, and decrease of lens potassium caused by anoxic atmospheres.

5. Ascorbic acid and ascorbic-dehydroascorbic acid at negative redoxes favored the nitrogen effect.

6. These observations are interpreted in terms of dehydroascorbic acid acting under nitrogen to allow the lenticular hydrogen transport process to occur and thus raising the production of metabolic energy to maintain weight and cation equilibrium.

University Hospitals.

We wish to thank Miss Louise Welter for assistance in part of the experimental work.

ADDENDUM

After presentation of this paper, it was noted that, in calculating the redox values in terms of Eh, the value for the calomel electrode had been neglected. As this is constant throughout all the experiments, the corrected redox values will be somewhat more positive in amount.

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Discussion

Dr. V. Everett Kinsey (Detroit, Michigan): I want to commend Dr. Schwartz and Dr. Leinfelder on the novelty of this approach to the subject. It is similar in a number of respects to some of the early work, I believe, of Thunberg, where methylene blue was used as a receptor, and may well lead to a better understanding of the reduction system which appears to operate in the lens, either with or without benefit of oxygen.

There are several points which occur to me. First, I think the evidence is weak that the ascorbic-acid level of the aqueous humor is affected with removal of the lens. We have recently published the results of some experiments in which it was shown that the ascorbic acid level in the aqueous humor in aphakic patients was normal. The earlier experimental results referred to by Dr. Schwartz disagree with ours primarily because the investigators did not take into account the fact that the ascorbic acid level in the aqueous humor is quite dependent on the blood level;

thus it is not possible to draw valid conclusions concerning the role of the lens in determining the ascorbic acid level of the aqueous humor unless the concentration in the blood is known.

Our results perhaps speak indirectly against the necessity for the functioning of dehydroascorbic acid normally, so far as metabolism is concerned.

Secondly, Dr. Schwartz has demonstrated that the metabolic pump action of the lens goes on in the presence of dehydroascorbic acid, even under anoxic conditions. The only experimental condition in which the redox potential was maintained normal was that in which dehydroascorbic acid was present in the presence of nitrogen. The potential was about 80 millivolts plus. From his work it appears that normally the lens functions in a medium having relatively well defined redox potential. Thus, one could argue with equal correctness that the lens function well at a redox potential of 80 millivolts because the redox potential is appropriate, rather than because of the presence of dehydroascorbic acid.

Thirdly, it seems to me it would be desirable to study the system under aerobic conditions where the redox potential is also maintained at the right level.

For instance, it may be that dehydroascorbic acid acts as a hydrogen acceptor under anoxic conditions but under aerobic conditions oxygen accepts the hydrogen.

One might suspect this, I think, when one considers that there is apparently a high concentration of cytochrome oxidase present in the epithelium of the lens; it seems to me unlikely that under these circumstances dehydroascorbic acid, rather than oxygen, would be the normal hydrogen acceptor.

Dr. Schwartz is aware of these possibilities, and further experimentation which they plan may well throw light on the situation.

DR. BERNARD SCHWARTZ (in closing): I wish to thank Dr. Kinsey for his comments. We apparently both are involved in the same process of trying to culture lenses. I would like, however, to reply to several of his comments.

One is the question of whether in aphakia the ascorbic acid level of the aqueous is decreased.

It seems—and he has brought out the point quite adequately—that there is no recognition taken of the ascorbic acid levels in plasma by some of the earlier workers. However, many of them are consistent in their observations regarding the difference of ascorbic-acid level between the aphakic and nonaphakic eye in the same animal. In the aphakic eye, the ascorbic-acid level is lower and the dehydroascorbic-acid level is higher. So, if we consider these results alone, perhaps, the results may be significant. This, I admit, is still a point of dispute, and, perhaps, further experiments could clarify this situation.

Secondly, he mentioned whether the redox potential alone, and not the ascorbic and dehydroascorbic acids were the important factor.

The reason we used ascorbic and dehydroascorbic acid was in order to create these redox potentials which occur in vivo and we felt were a product of ascorbic or dehydroascrobic acid or a mixture of both. Redox potentials may be created in other fashions; but since these relatively easily oxidized and reduced substances were in aqueous, they may be the source of the redox potentials. Nordmann's experiments pointed out this fact primarily back in the late thirties.

Whether, under an aerobic setup, the lens would prefer oxygen, I do not know. The reason we turned to ascorbic and dehydroascorbic acid was the fact that because of these relationships of avascular and vascular mass we thought the lens existed in a relatively anoxic state. I think further work should be done to determine whether this actually does occur in vivo, that is as to whether the lens needs more hydrogen acceptors than ordinarily supplied by oxygen.

I would also like to point out that we feel that the redox is a definite criterion in any culture work at all, including tissue culture and lens culture; and that perhaps the redox potential may be of significant value in Dr. Kinsey's culture media.

I noticed he used Dr. Parker's TC 199 culture media. In the November, 1954 *Canadian Journal of Biochemistry and Physiology*, Parker modified that media. I am sure he has modified it since then. At that time, he stated that he found that the redox potential was too high in TC 199; and he lowered it by adding large quantities of ascorbic acid.

So it may not necessarily be one compound or another compound or another one which we need to find to support growth; it may be a process such as oxidation reduction.

ANAEROBIC CARBOHYDRATE METABOLISM OF THE CRYSTALLINE LENS*

III. Triosephosphate, phosphoglycerate, and phosphoenolpyruvate

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In papers I and II[1] it was shown that cell-free extracts of crystalline lenses prepared from young rabbit eyes metabolized glucose, glucose-6-phosphate, fructose-6-phosphate, and fructose diphosphate to lactic acid in accordance with the Embden-Meyerhof glycolytic scheme.

The results to be reported establish triosephosphate, phosphoglycerate, and phosphoenolpyruvate as direct intermediates in the glycolytic pathway of lens metabolism. Furthermore, the generation of energy-rich phosphate associated with lens metabolism can be completely accounted for by the oxidative phosphorylation of triosephosphate and by the transfer of phosphate from phosphoenolpyruvate to adenine nucleotides.

Materials

The chemicals used were the same as previously described. In addition, triosephosphate was prepared according to the procedure of Meyerhof.[2] 3-Phosphoglyceric acid, barium salt, was purchased from the Schwarz Laboratories, Inc. Reduced diphosphopyridine nucleotide was prepared from chromatographically pure cozymase (Schwarz) according to the procedure of Lehninger.[3] Phosphoenolpyruvate, silver barium salt, was a generous gift from Dr. Erich Baer,† the barium salt was also purchased from the Bios Laboratories. Crystalline sodium pyruvate (Schwarz) and iodoacetic acid (Eastman Kodak) were used. Acetyl phosphate was prepared according to the procedure of Stadtman and Lipman.[4]

The following abbreviations are used: Fructose-diphosphate (FDP); hydrazine (Hy); iodoacetic acid (IAA); adenosine monophosphate (AMP); adenosine diphosphate (ADP); adenosin triphosphate (ATP); triosephosphate (TP); 3-Phosphoglyceric acid (PGA); Pyruvic acid (Pyr); reduced diphosphopyridine nucleotide (DPNH); phosphorus liberated by hydrolysis for seven minutes in 1 N HCl at 100°C. (7′P).

Analytic procedures

The analytic procedures employed were the same as previously described. In addition the hypoiodite method of Lohmann and Meyerhof[5] was used for the analysis of phosphoenolpyruvate. Triosephosphate was determined by the amount of alkali-labile phosphate according to the method of Baer and Fisher.[6] 1,3-Diphosphoglyceric acid was measured by the method of Lipmann and Tuttle[7] and calculated in terms of acetyl phosphate.

Experimental

Cell-free extracts of lenses were prepared and all incubations were conducted as previously described. In all cases, unless otherwise noted, the equivalent of one lens was used in each experiment.‡ In all incubations the main compartment of each flask contained the extract and KCl-$NaHCO_3$ buffer, while the side arm contained the other ingre-

* From the Wills Eye Hospital. This investigation was supported in part by a research grant-in-aid from the National Council to Combat Blindness and in part by a research grant from the National Institute of Neurological Diseases and Blindness, of the National Institutes of Health, Public Health Service.

† The solution prepared by removal of the silver and barium by means of NaCl and K_2SO_4 contained an impurity, probably traces of Ag^+, which interfered with lactic-acid determinations. For the latter incubations were run with the Bios preparation.

‡ The average dry weight content of the lens was 29 percent. In previous papers, because of a typographical error, the value was given as 20 percent.

TABLE 1

ACCUMULATION OF ALKALI-LABILE PHOSPHATE

Experiment	Additions	Lactic Acid μM	Alkali-labile P μM
24	FDP, AsO_4	15.67	0
24	FDP, AsO_4, IAA	1.47	4.55
29	FDP, PO_4	0.44	1.98
29	FDP, PO_4, Hy	0	5.88
29	FDP, PO_4, IAA	0	6.50

Total volume, 1.5 ml.; av. dry wt./lens, 40.2mg.; time, 60 mins.
Final concentrations: FDP, 3×10^{-3}M; PO_4, 6.60×10^{-3}M; Hy., 1.33×10^{-3}M; IAA, 1.58×10^{-2}M; AsO_4, 1.39×10^{-3}M.

dients. The final concentration of $NaHCO_3$ was 1.4×10^{-2}M, pH-7.4.

RESULTS

All values, unless otherwise indicated, have been calculated on the basis of 100 mg. dry weight of lens.

ACCUMULATION OF ALKALI-LABILE PHOSPHATE (table 1)

Lactic-acid formation from fructose diphosphate in the presence of arsenate was practically completely inhibited by iodoacetic acid (exp. 24) with the concomitant accumulation of triosephosphate measured as alkali-labile phosphate. Similar results were obtained in the presence of phosphate and also by the use of hydrazine, a trapping agent for carbonyl-containing compounds (exp. 29). An average of 5.64 μM of alkali-labile phosphate was formed which represents a conversion of 24 percent of the initial concentration of organic phosphate present in fructose diphosphate.

METABOLISM OF TRIOSEPHOSPHATE (table 2)

Quantitative amounts of lactic acid were produced by triosephosphate in a system fortified with arsenate or adenosine monophosphate (exp. 76). It can be seen that a good stoichiometric relationship exists between the amount of triosephosphate that disappeared and the amount of lactic acid formed. In the experiment with added adenosine monophosphate two moles of 7′P were generated for each mole of triosephosphate converted into lactic acid. Such was not the case with arsenate because of the ability of the latter to dissociate lactic acid production from the uptake of inorganic phosphate, as previously demonstrated. It is, moreover, clear that in the presence of AsO_4 the P of triosephosphate was converted to inorganic P, while in the presence of phosphate and adenosine monophosphate, the uptake of inorganic phosphate was equivalent to the triosephosphate P that disappeared. In the absence of arsenate and a suitable phosphate acceptor (exp. 38) the formation of lactic acid was markedly suppressed.

Experiments 74 and 71 show that iodoacetic acid not only inhibited the production of lactic acid but also prevented the generation of 7′P associated with the oxidation

TABLE 2

METABOLISM OF TRIOSEPHOSPHATE

Experiment	Additions	Lactic Acid μM	Alkali-labile P μM	7′P μM	Inorg. P μM
38	TP, PO_4	2.92	− 6.75	—	—
76	TP, AsO_4	10.30	− 9.78	—	+11.8
76	TP, PO_4, AMP	8.90	− 9.10	17.30*	− 7.6
74	TP, AsO_4, IAA	1.50	− 8.85	—	—
71	TP, PO_4, AMP, IAA	0	− 7.50	0	—
76F	TP, PO_4, AMP, NaF	2.46	−10.40	6.30*	—
76F-1	TP, PO_4, AMP, NaF, Pyr.	10.85	−10.30	10.80*	− 8.6

* Corrected for the 7′P of triosephosphate, 50% of which is 7′P.
Total volume 1.6 ml.; Av. dry wt./lens, 40.8 mg.; time, 60 mins.
Final concentrations: TP, 3.01×10^{-3}M; PO_4, 3.12×10^{-3}M; AMP, 3.12×10^{-3}M; NaF, 1.18×10^{-2}M; Pyruvate, 3.12×10^{-3}M; AsO_4, 2.97×10^{-3}M.

of triosephosphate and the reactions dependent thereon. The disappearance of alkali-labile P in the latter experiments could be accounted for by the instability of triosephosphate in prolonged contact with the slightly alkaline incubation mixture.

Experiment 76F shows that NaF markedly suppressed the formation of lactic acid and decreased the generation of 7′P by about one half, although it had no effect upon the disappearance of triosephosphate. The net result is the establishment of a ratio of 1:1* between the alkali-labile P disappeared and 7′P generated, instead of the previous ratio of 1:2 (exp. 76).

When pyruvate was added to such a system (exp. 76F-1) the production of lactic was restored to its original value and a stoichiometric relationship obtained between triosephosphate metabolized, 7′P generated, lactic acid produced and inorganic phosphate that disappeared. It is significant that the 7′P generated in the presence of fluoride from triosephosphate is one half that formed in its absence, for an equivalent amount of lactic acid produced and triosephosphate metabolized.

Metabolism of 3-phosphoglyceric acid (table 3)

It is clear that the metabolic transformation of phosphoglyceric acid to lactic acid is dependent upon the presence of reduced diphosphopyridine nucleotide (exp. 77), since in its absence, even with the addition of arsenate (exp. 37), no appreciable formation of lactic acid occurred. It is also apparent that in the presence of adenosine monophosphate 7′P was generated in an amount stoichiometrically equivalent to the quantity of lactic acid produced (exp. 77). The results of Experiments 78 indicate that in the presence of NaF the generation of 7′P from phosphoglyceric acid is completely suppressed while the reduction of added pryuvate to lactic acid in the presence of reduced diphosphopyridine nucleotide proceeded to completion. In the absence of reduced diphosphopyridine nucleotide pyruvate was not reduced to lactic acid (exp. 78F).

TABLE 3

Metabolism of 3-phosphoglyceric acid

Experiment	Additions	Lactic Acid μM	7′P μM	Inorg. P, μM
37	PGA, PO_4	1.20	—	—
37	PGA, AsO_4	1.14	—	—
77	PGA, AMP, DPNH	7.30	+7.35	+1.56
78	PGA, NaF	0.28	0	+2.75
78F	PGA, NaF, Pyr.	0.60	+0.49	+2.26
78F-1	PGA, NaF, Pyr., DPNH	11.18	0	+1.86

Total volume 1.7 ml.; Av. dry wt./lens, 44.8 mg.; time, 60 mins. Final concentrations: PGA, 3.08×10^{-3}M; PO_4, 4.4×10^{-4} M, AsO_4, 2.60×10^{-3}M; AMP, 3.76×10^{-3}M; NaF, 1.20×10^{-2} M; Pyruvate, 3.13×10^{-3}M; DPNH, 4.24×10^{-4}M.

Intermediate formation of phosphoenolpyruvate from phosphoglycerate

When phosphoglyceric acid (8.34×10^{-3}M) was incubated with lens extract in a total volume of 1.2 ml. in the absence of any additional cofactors, 6.53 μM of phosphoenolpyruvate was formed per 100 mg. dry wt. of lens in 60 minutes. This represents a conversion of 24 percent of the initial concentration of phosphoglycerate, the equilibrium value of which is about 29 percent.

Metabolism of phosphoenolpyruvate (table 4)

The results clearly indicate that phosphoenolpyruvate in a system fortified with added adenosine monophosphate and reduced diphosphopyridine nucleotide produced stoichiometric amounts of lactic acid and 7′P. That the generation of 7′P, under these conditions, is specifically associated with the metabolism of phosphoenolpyruvate is evident from the fact that pyruvate gave lactic acid but no 7′P; the presence of iodoacetic

* The deviation from the ratio of 1:1, as indicated in the table, is probably due to the fact that in the absence of a hydrogen acceptor, such as pyruvate, the reduced diphosphopyridine nucleotide formed with the oxidation of triosephosphate reduces part of the latter to glycerol-phosphate whose phosphate group is quite stable. This reduction, however, is not accompanied by the generation of 7′P. In the presence of pyruvate, as can be seen, the ratio is 1:1.

TABLE 4

METABOLISM OF PHOSPHOENOLPYRUVATE

Additions	7′P μM	Alkaline Iodine P μM	Lactic Acid μM
Phosphoenolpyruvate*	+8.42	−9.50	7.64†
Pyruvate	0.0	—	11.40

* Preparation of Baer.

† Determined in separate experiment in which a Bios preparation was used. Total volume, 1.7 ml.; Av. wt./lens, 42.9 mg.; time, 60 mins. All flasks contained: AMP, 3.53×10^{-3}M; NaF, 2.12×10^{-2}M; DPNH, 3.8×10^{-3}M; IAA, 1.76×10^{-3}M (latter added to prevent oxidation of triosephosphate, preformed or that formed during the reaction, which would generage 7′P.) In addition, phosphoenolpyruvate, 2.63×10^{-3}M; pyruvate, 2.94×10^{-2}M.

acid in the reaction mixture precluded the formation of 7′P by the oxidation of preformed triosephosphate. The presence of NaF had no apparent inhibitory effect upon the reaction.

COMPARISON OF AMP AND ADP AS PHOSPHATE ACCEPTORS (table 5)

In earlier publications it was shown that adenosine monophosphate was relatively more efficient as a phosphate acceptor than adenosine diphosphate in the metabolism of fructose diphosphate to lactic acid. With the identification of the two steps in which the 7′P is generated, it was of interest to determine the comparative efficiency of adenosine monophosphate and adenosine diphosphate, in each case, as phosphate acceptors. The results show that adenosine monophosphate permits the generation of approximately 30 percent more 7′P than does an equivalent concentration of adenosine diphosphate.

TABLE 5

COMPARISON OF ADENOSINE MONOPHOSPHATE AND AND ADENOSINE DIPHOSPHATE AS PHOSPHATE ACCEPTORS

A—*Fructose diphosphate*

Additions	7′P μM	Inorg. P μM
FDP, PO_4, NaF	+1.92	−2.49
FDP, PO_4, NaF, AMP	+8.75	−6.40
FDP, PO_4, NaF, ADP	+6.40	−6.50

Total volume, 1.5 ml; Av. dry wt./lens, 36.9 mg.; time, 60 mins. Final concentrations: FDP, 1.67×10^{-3}M; PO_4, 3.33×10^{-3}M; NaF, 1.0×10^{-2}M; AMP, 4.0×10^{-3}M; ADP, 4.0×10^{-3}M.

B—PHOSPHOGLYCERATE

Additions	7′P μM	Lactic Acid μM	Inorg. P μM
PGA, DPNH	+1.58	6.59	+5.34
PGA, DPNH, AMP	+10.66	13.0	+1.25
PGA, DPNH, ADP	+7.63	10.2	+2.71

Total volume, 1.5 ml.; Av. dry wt./lens, 38.6 mg.; time, 60 mins. Final concentrations: PGA, 3.33×10^{-3}M; DPNH, 3.78×10^{-3}M; AMP, 4.0×10^{-3}M; ADP, 4.0×10^{-3}M.

COMMENTS

The establishment of the pathways of metabolism fundamentally depends upon the demonstration that the presumed intermediates tend to accumulate when the sequence of enzymatic events is disrupted; the intermediates formed should serve as substrate precursors for the production of the end product of metabolism, in a more or less quantitative or stoichiometric manner. Although in this investigation the intermediary metabolites were not actually isolated and purified, the procedures employed to accumulate and detect the presumed intermediates have been universally accepted as providing good evidence for the presence of the indicated substances. In addition, pure crystalline compounds corresponding to the indicated intermediates were shown to behave in all respects as true substrates for production of lactic acid in the complete enzymatic system. As for the existence of a coenzyme as an integral component part of the metabolic pathway, it is necessary to demonstrate either an absolute need for its presence or that the enzymatic reaction proceeds faster and to a greater extent in its presence.

The enzymatic conversion of fructose diphosphate to triosephosphate by lens extract was established by showing that the latter accumulated in substantial quantities in the presence of hydrazine or iodoacetic acid. The formation of lactic acid was simultaneously inhibited (table 1).

Hydrazine traps the triosephosphate by forming a Schiff's base, while iodoacetic

acid inhibits the enzyme, triosephosphate dehydrogenase, and prevents the subsequent oxidation to phosphoglyceric acid. In the presence of a suitable phosphate acceptor, such as adenosine mono- or diphosphate, triosephosphate is converted stochiometrically and quantitatively to lactic acid with the concomitant development of two equivalents of energy-rich phosphate (table 2).

Iodoacetic acid inhibited both the metabolism of triosephosphate and the generation of 7′P. The presence of NaF similarly inhibited the production of lactic acid, whereas, iodoacetic acid completely inhibited the development of both equivalents of 7′P from triosephosphate, NaF suppressed the formation of only one equivalent of 7′P. Since the reduction of pyruvate to lactic acid occurred in the presence of the inhibitor (table 2), it is apparent that the site of fluoride inhibition is prior to the formation of pyruvate and does not prevent the oxidative phosphorylation of triosephosphates.*

Experiment 77 (table 3), indicates that phosphoglyceric acid in the presence of adenosine monophosphate (or adenosine diphosphate) and reduced diphosphopyridine nucleotide is readily converted into lactic acid with the concomitant formation of one equivalent of energy-rich phosphate. While the oxidation of triosephosphate is coupled with the uptake of inorganic phosphate (oxidative phosphorylation), the generation of 7′P from phosphoglyceric acid is not dependent upon the presence of inorganic phosphate. It is significant that this is the first instance, in these studies, in which the anaerobic carbohydrate metabolism of lens extracts showed a definite requirement for pyridine nucleotide. There is, apparently, sufficient oxidized pyridine nucleotide present in the lens extract preparation to permit the oxidation of triosephosphate to phosphoglyceric acid with the accompanying formation of reduced pyridine nucleotide.

This is borne out by the results of Experiment 76F-1 (table 2), which show that in the metabolism of triosephosphate in the presence of NaF, the addition of reduced pyridine nucleotide is not necessary for the reduction of the pyruvate to lactic acid. However, by starting with phosphoglyceric acid the oxidation step is eliminated and, with it, the formation of reduced pyridine nucleotide which is necessary for the reduction of pyruvate to lactic acid, as shown in Experiment 78F-1 (table 3). While NaF did not inhibit the oxidation of triosephosphate with the generation of one equivalent of energy-rich phosphate, it did prevent the formation of the second equivalent of 7′P associated with the metabolism of phosphoglyceric acid (tables 2 and 3).

Phosphoenolpyruvate was formed from phosphoglyceric acid in concentrations approaching the thermodynamic equilibrium value. The results (table 4) further indicate that the enzymatic breakdown of phosphoenolpyruvate produces 1.0 mole of energy-rich phosphate for each mole of lactic acid produced. It is this step which is the source of the second equivalent of 7′P generated in the metabolism of fructose diphosphate by lens extracts.

The results of our studies provide evidence that lens extracts utilize carbohydrates anaerobically in the production of lactic acid in accordance with the classical scheme of glycolysis described by Embden and Meyerhof. The essential enzymatic steps starting with fructose diphosphate are as follows:

1. Fructose diphosphate $\rightleftharpoons$ Dihydroxyacetone phosphate + Glyceraldehyde phosphate
2. Dihydroxyacetone phosphate $\rightleftharpoons$ Glyceraldehyde phosphate
3. Glyceraldehyde phosphate + PO_4 + DPN + ADP $\rightleftharpoons$ Phosphoglyceric acid + ATP + DPNH + H^+
4. Phosphoglyceric acid $\rightleftharpoons$ Phosphoenolpyruvate + H_2O
5. Phosphoenolpyruvic acid + ADP $\rightleftharpoons$ Pyruvic acid + ATP
6. Pyruvic acid + DPNH + H^+ $\rightleftharpoons$ Lactic acid + DPN

The incorporation of inorganic phosphate into ATP, equation 3, was shown by Warburg and Christian[9] to consist of two steps:

3(a) 3-Glyceraldehyde phosphate + DPN + PO_4 $\rightleftharpoons$ 1,3-diphosphoglyceric acid + DPNH + H^+

3(b) 1,3-diphosphoglyceric acid + ADP $\rightleftharpoons$ 3-Phosphoglyceric acid + ATP

* Meyerhof and Kiessling[8] found that fluoride inhibited the formation of phosphoenolpyruvate from phosphoglyceric acid by muscle and yeast extracts.

TABLE 6

FORMATION OF ACETYL PHOSPHATE

Experiment	Time minutes	Acetyl PO_4 formed		Average Lens Wt. (Dry) mg.
		μg-P/lens	% PGA	
B-7	60	36.0	11.6	44.8
B-6	60	33.4	10.8	40.6*
B-4	120	44.2	14.2	45.2
B-5	210	44.8	14.4	39.5

* Two lenses/flask used in this experiment.

Total volume, 1.8 ml. All flasks contained: PGA, 5.26×10^{-2}M; ATP, 5.26×10^{-3}M (7'P); Mg^{++}, 1.1×10^{-2}M; NaF, 1.1×10^{-2}M; H_2NOH, 5.26×10^{-1} M.

Attempts to demonstrate the formation of 1,3-diphosphoglyceric acid from fructose diphosphate or triosephosphate in the presence of hydroxylamine were unsuccessful. However, by approaching the reaction from the right side, that is, starting with phosphoglyceric acid and adenosine triphosphate, as was done by Lipmann and Tuttle[7] with extracts of cat muscle and by Holloway[10] with extracts of pea meal, it was found (table 6) that 11 to 14 percent of the phosphoglyceric acid was converted to 1,3-diphosphoglyceric acid measured in terms of acetyl phosphate by the hydroxylamine-ferric chloride method of Lipmann and Tuttle.

SUMMARY

Cell-free extracts of crystalline lenses prepared from young rabbit eyes were incubated at 37°C. under anaerobic conditions with the following phosphate esters: fructose diphosphate, triosephosphate, 3-phosphoglyceric acid, and phosphoenolpyruvic acid. In properly fortified systems all esters produced practically quantitative amounts of lactic acid. The esters were further identified as intermediary metabolites by their tendency to accumulate when the actively metabolizing system was interrupted by means of iodoacetic acid or hydrazine (triosephosphate), sodium fluoride (phosphoglyceric acid), or under conditions of phosphate acceptor deficiency (phosphoenolpyruvic acid). The enzymatic reduction of pyruvic acid to lactic acid was shown to depend upon the presence of reduced diphosphopyridine nucleotide. The generation of energy-rich phosphate was shown to be associated with the oxidative phosphorylation of triosephosphate and with the transphosphorylation from phosphoenolpyruvate to adeninenucleotide. Adenosine monophosphate is a somewhat more efficient phosphate acceptor than adenosine diphosphate. The pathway of anaerobic carbohydrate metabolism by lens extracts is essentially as described by the Embden-Meyerhof scheme of glycolysis.

1601 Spring Garden Street (30).

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Discussion

Dr. John E. Harris (Portland, Oregon): This is the third paper which these authors have presented to this group in a series concerning the metabolic steps by which the energy of the lens is produced. They have approached this significant question in a very logical and step-wise fashion, setting out first to determine whether anaerobic glycolysis of the lens follows the usual Embden-Meyerhof sequence. That it does so, they have proved quite conclusively.

To achieve such proof they have employed a cell-free extract of lens homogenate and studied the fate of various intermediates in the Embden-Meyerhof system. In their first paper, they began with glucose and glucose-6-phosphate; in the second paper, they demonstrated the ability of the lens extract to utilize fructose-1, 6-diphosphate; now, using various three-carbon intermediates further down the chain, they have added final proof that anaerobic glycolysis in the lens follows the Embden-Meyerhof pathway. In the course of these studies, they have demonstrated the production of high-energy phosphates, the compounds which provide the immediate energy for most cellular processes requiring work.

Anaerobic glycolysis of the lens cannot supply sufficient energy to meet its requirements. That oxygen is necessary for the normal function of the lens has been demonstrated in many laboratories. I thought, perhaps, I might quickly review some of the possibilities by which an oxidative process involving oxygen might provide more high-energy phosphate.

Among the possibilities is the utilization of three-carbon residues via the Krebs cycle. That this occurs in the lens has never been proved conclusively. Using cation transport as a measure of lens vitality, we have been unable to demonstrate that either pyruvic acid, lactic acid, or certain compounds of this cycle serve as a substrate in the lens. Since such experiments employ the intact lens, the negative results may simply indicate that the added substance does not diffuse readily into the lens. In this respect a cell-free extract (such as Dr. Green and associates employed) is more ideal. On the other hand, destruction of the organization of the lens may yield misleading information. The question of energy production in the lens via the Krebs cycle, then, is not fully settled.

One step which I think merits consideration is the oxidation of reduced DPN to the oxidized form. If this occurs via the cytochrome system (with the ultimate use of oxygen), three molecules of ATP are formed for each three-carbon residue utilized. Therefore, six molecules of ATP would be formed if glucose were the starting substrate. If such were the case, pyruvic acid would not be reduced to lactic acid. The fact that lactic acid is found in high concentration in the lens therefore mitigates against this view.

Another possibility worth while mentioning is the so-called hexosemonophosphate shunt (sometimes called the Warburg-Dickens-Lipmann shunt). The lens was shown to be capable of utilizing this pathway for the metabolism of glucose by Kinoshita. (Slide) Here is the classic system which Dr. Green and associates have shown actually to prevail; here we note the shunt which merges with the classic system at the glyceraldehyde stage. It requires TPN at two different points, as can be seen. It is not known that this will necessarily give more ATP. The oxidation of TPN might possibly increase the yield of ATP.

Dr. Carl Wachtl (Detroit, Michigan): I think Dr. Green has very well demonstrated the glycolytic scheme taking place in lens homogenates.

He uses adenosine monophosphate (AMP) as a phosphate acceptor; and I wonder whether he has also tried to use adenosine diphosphate (ADP). We have adenosine triphosphate (ATP) in our culture medium and I have been wondering whether an additional phosphate acceptor may be required.

Ordinarily, the phosphate acceptor used is ADP. There is also a reaction known to occur between AMP and ATP to give two molecules of the ADP. I wonder whether this is what happens in this case.

Dr. Green probably adds enough ATP in his system to make up for the deficiency in ADP if this reaction does occur.

Dr. V. Everett Kinsey (Detroit, Michigan): I think it would be worth while to make a similar study of lenses from which the epithelium and capsule had been removed. This would make it possible to separate the contribution toward formation of high-energy phosphate of the latter portions of the lens from that of the cortex and nucleus.

Dr. Harry Green (in closing): It is always a distinct pleasure to have a paper discussed by Dr. Harris.

In answer to Dr. Wachtl's question concerning the fact that ADP usually is the phosphate acceptor in such systems, it is the same question Dr. Dische asked last year in San Francisco. The answer at that time was that adenosine monophosphate is apparently a more effective acceptor in our system.

Recently, we had occasion to test the relative efficiency of ADP and AMP as phosphate acceptors in the two steps where seven-minute phosphate is generated; namely, in the oxidation of triosephosphate and transphosphorylation from phosphopyruvate. In both cases, we found that AMP is approximately 30 percent more effective than ADP as a phosphate acceptor in lens extracts.

With regard to the possible presence of myokinase in lens extracts being responsible for the observed effect, we have not attempted to assay for this enzyme. However, in view of results with stoichiometric quantities of the two adenosine phosphates, it is doubtful whether the stimulation of glycolysis by adenosine monophosphate is dependent upon the possible presence of myokinase.

Dr. Kinsey's suggestion that we strip the epithelium off the lens and try to isolate exactly where this thing is taking place is a very good suggestion.

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TWENTY-FOURTH MEETING

of the

Association for Research in Ophthalmology, Inc.

Proceedings

Business Session — Former Secretaries
Auditors' Report — Directory of Members
Committees — New Members
Former Trustees — Geographic List
Proctor Medal Recipients

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Atlantic City, New Jersey

June 7, 8, and 9, 1955

BUSINESS MEETING

The opening session of the 24th meeting of the Association for Research in Ophthalmology, at Atlantic City, New Jersey, June 6-10, 1955, convened at 1:20 p.m. in the St. Dennis Room of the Dennis Hotel, Dr. William F. Hughes, Jr., Chicago, Illinois, Chairman of the Board of Trustees, presiding.

The annual business meeting was held on June 7th, Dr. William F. Hughes, Jr., presiding.

Upon motion of Dr. J. H. Allen, New Orleans, Louisiana, which was duly seconded, it was voted unanimously that the dues be raised for educational members from $2.00 to $3.00 per year; and, for active members, from $5.00 to $10.00.

Upon motion regularly made and seconded, it was voted unanimously that Dr. Parker Heath, Dr. Lawrence T. Post, and Dr. Alan C. Woods be made honorary members.

Upon motion regularly made and seconded, it was voted unanimously that Dr. Hans Barkan be elected to life membership.

Upon motion of Dr. Allen, which was duly seconded, it was voted unanimously to adopt the report of the Nominating Committee, as follows:

Member of Board of Trustees: Dr. Jonas S. Friedenwald, Baltimore, Maryland.

Secretary-Treasurer: Dr. Lorand V. Johnson, Cleveland, Ohio.

The following announcement was read, with congratulations from the Board of Trustees:

THE AMERICAN JOURNAL OF OPHTHALMOLOGY will soon publish a special section to be called "Ophthalmic Research." The abstracts of papers presented at the various sectional meetings of the Association for Research in Ophthalmology will be published, together with the abstracts of papers concerning research in ophthalmology presented at various local meetings throughout the country. News items concerning the sources of research grants, application deadlines, recipients of research support, appointees to national research groups, and similar items will be published.

The column will be edited by Dr. Frank W. Newell, 950 East 59th Street, Chicago 37, Illinois.

Each section secretary will be responsible for the solicitation of abstracts and should submit them to the editor as early as possible, but no later than at the conclusion of each sectional meeting.

SUMMARY OF MEMBERSHIP BY YEARS
to December 31, 1954

Years Ended December 31	Total Members	Years Ended December 31	Total Members
1954	[1]815	1941	279
1953	[2]713	1940	270
1952	[2]672	1939	268
1951	[2]556	1938	272
1950	[2]509	1937	249
1949	[2]474	1936	240
1948	422	1935	245
1947	306	1934	230
1946	324	1933	219
1945	[3]—	1932	203
1944	283	1931	193
1943	[3]—	1930	134
1942	281		

[1] Represents official membership, whether dues were paid or unpaid for current and prior years.
[2] Includes only members whose dues were paid in full to date.
[3] Not available, due to wartime dislocation.

AUDITORS' REPORT

Association for Research in Ophthalmology, Inc.

To the Members of the Board of Trustees
Association for Research in Ophthalmology, Inc.
New Orleans, Louisiana

Gentlemen:

Scope of Examination

We have examined the cash basis accounts of the secretary-treasurer of the Association for Research in Ophthalmology, Incorporated, as of December 31, 1954, and the recorded transactions for the year then ended. Our examination was made in accordance with generally accepted auditing standards applicable to cash basis accounting and accordingly included such tests of the accounting records and other such auditing procedures as we considered necessary in the circumstances, except that we did not confirm unpaid dues directly with members.

History

The association was incorporated on July 20, 1936, under the laws of the state of New York. However, it had been an unincorporated group for some years earlier, operating under a constitution and related bylaws which were embodied in the certificate of incorporation. The corporation has no shareholders and is exempt from federal, state, and local taxes. However, it is required to file a federal information return annually, reporting the source and disposition of income.

Comments

At the meeting of the board of trustees in June, 1952, the board directed that the $250.00 annual interest income from the Proctor medal fund be deposited henceforth in the general fund and that the cost of the annual Proctor medal be paid out of the general fund.

According to information given us orally by the secretary-treasurer, it is the intention of the board of trustees, governing body of the association, to retain members whose dues are delinquent until the board itself orders that they be dropped for nonpayment. The constitutional provision remains unchanged which states that new members, upon being approved by the admissions committee and the board of trustees, must pay dues to attain membership.

At its meeting in May, 1953, the board of trustees directed that members whose dues were in arrears be notified and that if payment was not forthcoming they be dropped from the rolls. On December 31, 1954, past due memorandums were mailed by the secretary-treasurer to all members who were delinquent in paying dues for 1953 or prior years. We were advised that 1954 delinquents were advised thus only if they were also delinquent for earlier years.

We did not verify by direct communication with members the dues which were delinquent. According to the records of the association, the entries therein showed 202 delinquencies as of December 31, 1954. If any members carried as delinquent had paid before that date and payments had been lost in the mails before recordation, our examination might not have detected the omission.

All individual membership cards were examined and listed by us and their totals by classes of membership related to the recorded cash receipts for the period.

We examined minutes of the board of trustees, constitution, and other pertinent data.

The records were found to be well kept and the financial transactions of the year properly authorized. Please accept our sincere thanks for the courtesy and assistance given us during the examination.

Certificate

In our opinion, subject to the limitation that we did not confirm dues in arrears directly with members, the accompanying statements present fairly the fund balances of the Association for Research in Ophthalmology, Incorporated, as of December 31, 1954, and the receipts and disbursements for the year then ended, based on the recorded cash transactions. The statements conform to generally accepted cash basis accounting principles applied on a basis consistent with that of the preceding year. We do not feel that the exception referred to above is sufficiently important to negate this opinion.

Very truly yours,

B. B. Woolley & Co.,
New Orleans, Louisiana,
February 23, 1955.

Cash and Securities in Funds

December 31, 1954

	General Fund	Proctor Medal Fund	Total
Cash:			
Cash on hand	$ 259.00	$ —	$ 259.00
Cash in bank	1,786.97	15.37	1,802.34
	2,045.97	15.37	2,061.34
Securities:			
U. S. Treasury bonds[1]	—	10,184.63	10,184.63
	$ 2,045.97	$10,200.00	$12,245.97

[1] At cost—2½%—due 1967-72. At December 31, 1954, coupons amounting to $125.00, matured in 1954, were affixed to the bonds and were detached and deposited in bank in the presence of the auditors.

Statement of Cash Receipts and Disbursements

Year Ended December 31, 1954

		General Fund	Proctor Medal Fund
Cash Balance January 1, 1954		$1,608.58	$15.37
Add—Receipts:			
Prior years' dues:			
47 Active members @ $5.00 each	$235.00		
5 Educational members @ $2.00 each	10.00		
2 Sustaining members @ $25.00 each	50.00	295.00	
1954 Dues—(Page 6)		3,774.00	
1955 Dues—2 active members		10.00	
1955 Dues—1 educational member		2.00	
Banquet proceeds		265.00	
Bond interest		375.00	
Total Receipts		4,721.00	—0—
		6,329.58	15.37

Deduct—Disbursements:			
Convention expenses:			
Dinners	486.83		
Programs, mailing fees, notices	346.93		
Expenses—Secretary-Treasurer	250.00	1,083.76	
Publication—American Journal of Ophthalmology[3]		1,000.00	
Stationery, supplies and printing		918.46	
Auditing		153.00	
Postage and express		85.43	
Insurance—$5,000.00 position bond—Secretary-treasurer		25.00	
Safety box rental		8.80	
Telephone		6.93	
Salaries—Gross[1]	550.00		
Less withholding and FICA taxes withheld	110.00	440.00	
Withholding and FICA taxes paid		133.50	
Proctor Medals[2]		290.60	
Bank charges		6.35	
Contributions		25.00	
Sundry		10.20	
Gift (to former Chairman of Board of Trustees)		96.58	
Total Disbursements		4,283.61	—0—
Cash Balance December 31, 1954		$2,045.97	$15.37
Composed of:			
Cash in bank December 31, 1954		$1,786.97	$15.37
Cash on hand—subsequently deposited		259.00	—
		$2,045.97	$15.37

[1] Salary for December, 1954, was paid in January, 1955.
[2] Includes Proctor Medals for both 1954 and 1955.
[3] Publication of 1953 meeting.

Changes in Membership and Reconciliation with Dues Paid

Year Ended December 31, 1954

Changes in Membership	Inactive	Life	Honorary	Educational	Active	Sustaining	Total
Membership—January 1, 1954	1	1	12	80	587	32	713
Add:							
Elected for 1954 membership at October, 1953, meeting of Board of Trustees				4	15		19
Elected for 1954 membership at June, 1954, meeting of Board of Trustees				14	80		94
Total Additions	—0—	—0—	—0—	18	95	—0—	113
Deduct:							
Resigned in 1954					4		4
Deceased in 1954					7		7
Total Deductions					11		11
Changes in Classes of Memberships:							
From active to sustaining							
From sustaining to active					8	(8)	
From educational to active				(11)	11		
From active to inactive	1				(1)		
Net Changes	1			(11)	(8)	18	—0—
	2	1	12	87	663	50	815
Unaccounted for differences				(2)	2		
Membership December 31, 1954	2	1	12	85	665	50	815
Reconciliation with Dues Paid							
Dues Paid in 1953 for 1954					3		3
Dues paid in 1954 for 1954:							
Inactive, life and honorary—Waived	2	1	12				15
Educational @ $2.00 each $74.00				37			37
Active @ $5.00 each $2,550.00					510		510
Sustaining @ $25.00 each 1,150.00						46	46
$3,774.00	2	1	12	37	513	46	611
Add:							
Unpaid membership Dec. 31, 1954				50	148	4	202
	2	1	12	87	661	50	813
Unaccounted for differences				(2)	4		2
	2	1	12	85	665	50	815

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